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**Activation and Role of Memory CD8 T Cells in Heterologous
Antiviral Immunity and Immunopathology in the Lung**

A Dissertation Presented

By

Hong Chen

**Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of**

DOCTOR OF PHILOSOPHY

December 9, 2002

IMMUNOLOGY AND VIROLOGY

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**Activation and Role of Memory CD8 T Cells in Heterologous Antiviral
Immunity and Immunopathology in the Lung**

**A Dissertation by
Hong Chen**

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ABSTRACT

Each individual experiences many sequential infections throughout the lifetime. An increasing body of work indicates that prior exposure to unrelated pathogens can greatly alter the disease course during a later infection. This can be a consequence of a phenomenon known as heterologous immunity. Most viruses invade the host through the mucosa of a variety of organs and tissues. Using the intranasal mucosal route of infection, the thesis focused on studying modulation of lymphocytic choriomeningitis virus (LCMV)-specific memory CD8 T cells upon respiratory vaccinia virus (VV) infection and the role of these memory CD8 T cells in heterologous immunity against VV and altered immunopathology in the lung.

The VV infection had a profound impact on memory T cells specific for LCMV. The impact included the up-regulation of CD69 expression on LCMV-specific CD8 memory T cells and the activation of their *in vivo* IFN- γ production and cytotoxic function. Some of these antigen-specific memory T cells selectively expanded in number, resulting in modulation of the original LCMV-specific T cell repertoire. In addition, there was a selective organ-dependent redistribution of these LCMV-specific memory T cell populations in secondary lymphoid tissue (the mediastinal lymph node and spleen) and the non-lymphoid peripheral (the lung) organs. The presence of these LCMV-specific memory T cells correlated with IFN- γ -dependent enhanced VV clearance, decreased mortality and marked changes in lung immunopathology. Thus, the participation of pre-existing memory T cells specific for unrelated agents can alter the dynamics of mucosal immunity. This is associated with an altered disease course in response to a pathogen.

The roles for T cell cross-reactivity and cytokines in the modulation of memory CD8 T cells during heterologous memory CD8 T cell-mediated immunity and immunopathology were investigated. Upon VV challenge, there were preferential expansions of several LCMV-specific memory CD8 T cell populations. This selectivity suggested that cross-reactive responses played a role in this expansion. Moreover, a VV peptide, partially homologous to LCMV NP 205, stimulated LCMV-NP205 specific CD8 T cells, suggesting that NP205 may be a cross-reactive epitope. Poly I:C treatment of LCMV-immune mice resulted in a transient increase but no repertoire alteration of LCMV-epitope-specific CD8 T cells. These T cells did not produce IFN- γ *in vivo*. These results imply that poly I:C, presumably through its induced cytokines, was assisting in initial recruitment of LCMV-specific memory CD8 T cells in a nonspecific manner. VV challenge of LCMV-immune IL-12KO mice resulted in activation and slightly decreased accumulation of LCMV-specific CD8 T cells. Moreover, there was a dramatic reduction of *in vivo* IFN- γ production by LCMV-specific IL-12KO CD8 T cells in the lung. I interpreted this to mean that IL-12 was important to augment IFN- γ production by memory CD8 T cells upon TCR engagement by antigens and to induce further accumulation of activated memory CD8 T cells during the heterologous viral infection.

This thesis also systematically examined what effect the sequence of two heterologous virus challenges had on viral clearance, early cytokine profiles and immunopathology in the lung after infecting mice immune to one virus with another unrelated viruses. Four unrelated viruses, [LCMV, VV, influenza A virus or murine cytomegalovirus (MCMV)], were used. There were many common changes observed in the acute response to VV as a consequence of prior immunity to any of three viruses,

LCMV, MCMV or influenza A virus. These included the enhanced clearance of VV in the lung, associated with enhanced T_H1 type responses with increased IFN- γ and suppressed pro-inflammatory responses. However, immunity to the three different viruses resulted in unique pathologies in the VV-infected lungs, but with one common feature, the substitution of lymphocytic and chronic mononuclear infiltrates for the usual acute polymorphonuclear response seen in non-immune mice. Immunity to influenza A virus appeared to influence the outcome of subsequent acute infections with any of the three viruses, VV, LCMV and MCMV. Most notably, influenza A virus-immunity protected against VV but it actually enhanced LCMV and MCMV titers. This enhanced MCMV replication was associated with enhanced T_H1 type response and pro-inflammatory cytokine responses. Immunity to influenza A virus appeared to dramatically enhance the mild lymphocytic and chronic mononuclear response usually observed during acute infection with either LCMV or MCMV in non-immune mice, but LCMV infection and MCMV infection of influenza A virus-immune mice each had its own unique features. Thus, the specific sequence of virus infections controls the outcome of disease.

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ABBREVIATIONS

aa	amino acid
AMI	acute mixed infiltrates
APC	antigen-presenting cell
BAL	bronchial alveolar lavage
BALT	Bronchus-associated lymphoid tissue
BCG	bacillus Calmette-Guerin
BO	bronchiolitis obliterans
Ca	calcium
CCR7	CC chemokine receptor 7
CFSE	carboxyfluorescein succinimidyl ester
CMI	chronic mononuclear infiltrates
CMV	cytomegalovirus
cpm	counts per minute
CTL	cytotoxic T lymphocytes
CXCR	CXC chemokine receptor
E:T	effector to target ratio
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbant assay
FasL	Fas ligand
FCS	fetal calf serum

Flu	influenza A virus
GALT	gut-associated lymphoid tissue
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GP	glycoprotein
H+E	hematoxylin and eosin
HA	hemagglutinin
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV	herpes simplex virus
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
ICOSL	immunocostimulatory signaling ligand
IE	immediate early
IFN- γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
kD	kilo Dalton
KO	knock-out
LAT	linker for activation of T-cells
LCMV	lymphocytic choriomeningitis virus
LDA	limiting dilution assay

M	matrix
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MCMV	murine cytomegalovirus
MCP	monocyte chemoattractant protein
MEKK	MAPK extracellular signal-regulated kinase kinase
MFI	mean fluorescent intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLN	mediastinal lymph node
MN	mononuclear
MOI	multiplicity of infection
mRNA	messenger RNA
NA	neuraminidase
NB	necrotizing bronchiolitis
ND	not done
No.	number
NOD	non-obese diabetic
NP	nucleoprotein
pCTL	CTL precursors
PFU	plaque-forming unit
PMN	polymorphonuclear

Poly I:C	poly inosinic/cytidylic acid
PV	Pichinde virus
RSV	respiratory syncytial virus
TCR	T cell receptor
T _H	T help type
TMEV	Theiler's murine encephalomyelitis virus
TNF	tumor necrosis factor
VSV	vesicular stomatitis virus
VV p1	vaccinia virus peptide 1
VV	vaccinia virus
WT	wild type

CHAPTER I

INTRODUCTION

A. Heterologous immunity and variability in pathogenesis of infections with microorganisms. Infections with microorganisms can run markedly different courses in different individuals, ranging from subclinical to clinical or nonlethal to lethal. This variability in responsiveness is related to the dose of pathogen, its route of infection, as well as the genetics, physiological state, and immune status of the host. Heterologous immunity, or protection by one invading organism against another unrelated pathogen, has been recognized in bacterial infections and parasitic malaria infections for decades (Clark et al., 1976; Cox, 1970). However, in those cases, heterologous immunity has been often referred to as a phenomenon occurring between concomitant infections, in which pro-inflammatory cytokines and nitric oxide produced by macrophages are important players (Clark, 2001). Another situation, different from concomitant infections, is when individuals experience many sequential infections throughout their lifetime. Until recently, research into anti-viral immune responses in animal models has focused on studying hosts that were infected with one virus and sheltered from other environmental pathogens. However, evidence is gradually accumulating to suggest that prior immunity to heterologous pathogens can have a significant impact on the outcome of each new infection. An example of this in human disease is dengue fever, for which a severe shock syndrome can arise if a host that has been exposed previously to one of the four dengue-virus serotypes is later exposed to a second serotype (Halstead, 1989; Mathew et al., 1998). The earlier work conducted in our laboratory has clearly shown with intraperitoneal

(i.p.) systemic viral infections that memory T cells generated in response to one virus can indeed alter the course of disease caused by unrelated viruses (Selin et al., 1998). This phenomenon has been termed T cell-mediated heterologous immunity.

B. The biology, genetics and pathogenesis of LCMV, VV, influenza virus and MCMV. Infections of mice with lymphocytic choriomeningitis virus (LCMV), vaccinia virus (VV), influenza A virus or murine cytomegalovirus (MCMV) have proven to be some of the most important model systems for the study of the immune response to viral infections and viral diseases in human. Research presented in this thesis utilized the mouse models of infections with these four viruses to study heterologous immunity.

Studies in the model of LCMV infection have laid the basis for many seminal concepts of immunology, the discovery of two of which resulted in the 1960 and 1996 Nobel prizes in Medicine (Burnet FM, 1949; Zinkernagel and Doherty, 1974). LCMV is the prototype virus of the arenavirus family, which consists of ambisense enveloped RNA viruses. Of the two ambisense RNAs of LCMV, the small 23S S RNA encodes the 63-kd nucleoprotein (NP) and the 75-kd glycoprotein, which is cleaved into the virion surface glycoproteins GP1 and GP2 (Buchmeier et al., 1978). The larger 31S L RNA encodes a 200-kd L protein, which serves as an RNA-dependent RNA polymerase, and a small 11-kd zinc binding Z protein (Salvato et al., 1992; Salvato and Shimomaye, 1989). Alpha-dystroglycan has recently been identified as the receptor for LCMV and some other arenaviruses (Cao et al., 1998). This ubiquitous receptor allows LCMV to infect a wide variety of hosts, including mice, humans, chimpanzees, and hamsters. The LCMV infection is relatively non-cytopathic, and the LCMV infection in human can either be subclinical or else present as a nonmeningeal gripe-like ailment or a moderate to

severe meningitis (Welsh, 2000). Several strains have been isolated for laboratory use since the 1930's. The strain used most in this thesis is the Armstrong strain, which was originally derived from a monkey that was suffering from lymphocytic choriomeningitis. Infection of adult immunocompetent mice with strain Armstrong leads to viral clearance and life long immunity. Occasionally used in this thesis is the clone 13 variant that differs from the Armstrong strain by only two amino acids (Salvato et al., 1991). These changes result in an increased ability to infect macrophages and increased replication in infected cells (Matloubian et al., 1993).

VV is a member of the family *Poxviridae*, which comprises a group of complex animal DNA viruses (Witteck, 1999). Poxviruses are among the largest and most complex viruses known. VV virions contain a large double-stranded DNA genome that consists of about 192 kb. The DNA sequence of the viral genome provides evidence for the presence of about 200 potential protein-coding sequences. The VV genome encodes many enzymes and structural proteins for its infection cycle. VV is cytolytic, has a very broad host range and can infect most vertebrate animals. Although VV is not associated with a naturally occurring disease in humans, it is used as a smallpox vaccine and the serious complications of smallpox vaccination, a phenomenon called progressive vaccinia, occur at very low frequency. The strain used in this thesis is the Western Reserve (WR) laboratory strain.

The highly contagious acute respiratory illness known as influenza has afflicted humans since ancient times (Webster, 1999). Influenza A virus belongs to the *Orthomyxoviridae* family of RNA viruses. It has eight segmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. There are

hemagglutinin (HA), enzyme neuraminidase (NA) and ion channel M2 proteins in the envelope. Beneath the lipid envelope is a membrane or matrix (M) protein surrounding a helical complex containing the nucleoprotein (NP) and three polymerase proteins. Influenza A virus commonly causes disease in humans, pigs, horses, and occasionally in birds. It can be also adapted to experimental animals such as mice. The virus replicates in the mouse upper and lower respiratory tract and, after adaptation, can cause pneumonia and death. The strain used in this thesis is A/PR/8/34 (H1 N1).

CMVs, the prototype members of the β -herpesvirus subfamily of the *Herpesviridae* family, are widely distributed in nature. Many animal species have an associated CMV which exhibits exquisite species-specificity. These viruses infect for life and are readily isolated from salivary gland explants. The importance of CMV as a human pathogen has increased, as immunosuppressive post-transplant therapies and AIDS, as well as other immunodeficiency states, have come to the forefront of medicine. MCMV is a surrogate for studying human CMV-associated pathogenesis (Staczek, 1999). DNA sequencing of the Smith strain (used in this thesis) genome reveals 230 kb. Like VV, MCMV encodes its own enzymes and structural proteins for its infection cycle. Infected cells display a cytopathology that is characterized by the enlargement (cytomegaly) of the cell. Strain variation influences disease presentation and recovery. Most laboratory strains of MCMV are descendents from the original Smith strain isolated in 1934. Immunocompetent mice manifest minor illness when inoculated with a sublethal dose. Resolution of the productive infection need not clear virus from organs such as salivary glands, which become chronically infected in either a persistent or latent manner.

C. The host immune responses to viral infections and virus-induced immunopathology.

During the early stages of infection with viruses such as LCMV, VV, influenza A virus or MCMV, various components of host natural immunity play roles in maximizing the effectiveness of the natural immune system and initiating the specific immunity against viruses. The components of host natural immunity include humoral factors (e.g. natural antibodies and complement), preexisting and inducible anti-viral cytokines and chemotactic factors (e.g. type I and II interferons, IL-1, IL-6, TNF- α , IL-12, MIP, MCP, and IL-8) produced by virus-infected macrophages or other cells, and peptide defensins made by neutrophils, as well as other components of the acute phase response (Welsh and Sen, 1997). Natural killer (NK) cells represent an early host response mechanism particularly suited to control viral infections such as MCMV (Bukowski et al., 1984). $\gamma\delta$ T cells also play a role against viruses such as VV, HSV and influenza A virus (Carding et al., 1990; Rakasz et al., 1999; Selin et al., 2001; Welsh and Sen, 1997). Of note, there are redundancies of the components of natural immunity. Different effector systems may have greater or lesser significance against a given virus. In addition, the rapidly mobilized response by memory cytotoxic T lymphocytes (CTLs) may be able to contribute to the natural resistance against virus by more specific mechanisms. This will be one of the major topics in this thesis (see below).

Viral infections are dealt with largely by the specific adaptive humoral and T cell-mediated immune responses, in which effector T lymphocytes and secreted immunoglobulin (Ig) molecules eliminate infected cells and neutralize free virions, respectively (Doherty and Ahmed, 1997). Continuous antibody production lasts for months (influenza A virus, up to 1 year in human) to many years (VV, up to 30 years in

human). In the long term, LCMV-specific plasma cells are found at highest frequency in mouse bone marrow (Slifka et al., 1995). However, LCMV-specific neutralizing antibodies are generated rather late, i.e., after 1 to 3 mo (Battegay et al., 1993b). Upon secondary viral infection, pre-existing antibody provides the first line of defense against reinfection (Thomsen and Marker, 1988; Wells et al., 1979). CD4 T cells are potent regulators of the immune system as a consequence of the cytokines they produce. During the acute infection with a virus, CD4 T cells may act as effector cells through the secretion of anti-viral cytokines such as IFN- γ and TNF- α (Biron, 1994; Doherty et al., 1997; Lucin et al., 1992; Zinkernagel, 1996). Providing B cells with the help necessary to produce anti-viral antibodies is one of the primary roles CD4 T cells play during virus infections (Bachmann and Zinkernagel, 1996; Doherty et al., 1997; Graham and Braciale, 1997; Jonjic et al., 1989). The role of CD4 T cells in the induction of the virus-specific CD8 T cell response varies between experimental systems. CD8 CTL responses to VSV, certain subtypes of influenza A virus, and VV are significantly diminished in mice that lack CD4 T cells (Battegay et al., 1996; Leist et al., 1989; Tripp et al., 1995b), whereas the CD8 T cell response to an acute LCMV infection seems to be less dependent on CD4 T cells (Ahmed et al., 1988; Nahill and Welsh, 1993). LCMV, influenza A virus, CMV and VV, like many other viruses, are potent inducers of CD8 T cell responses. These virus-specific CTL responses are important for clearance of these viruses and recovery from infection (Byrne and Oldstone, 1984; Flynn et al., 1998; Koszinowski et al., 1987; Spriggs et al., 1992). Increased frequency of virus-specific memory T cells and ability of these cells to rapidly proliferate and develop into effector T cells provides a highly effective second line of defense against re-infection. This will be one major object of this thesis.

Although viruses like VV, influenza A virus, and MCMV can cause disease directly through their cytopathic effects, which lead to cytolysis, necrosis or apoptosis of host target cells, protection by immune mechanisms is often accompanied by immunopathology. For example, during a viral infection, the inflammatory responses induced by components of the innate and adaptive immune systems play roles in defense against viruses but also contribute to many of the symptoms of disease. Although LCMV is relatively non-cytopathic, the LCMV-induced potent T cell responses, which are importance to control viral clearance, cause severe immunopathology in targeted organs of their host (Zinkernagel and Doherty, 1975). This will be another major topic of this thesis.

D. Antigen recognition and activation of T cells. T cells play a central role in the immune response to many virus infections (Doherty et al., 1992; Zinkernagel et al., 1996). The majority of mature T cells express a T cell receptor (TCR), which is a membrane-bound heterodimer composed of α and β chains (Fields and Mariuzza, 1996). T cells that express α/β TCR can be divided into cells expressing either CD8 or CD4 surface antigens. T cells must have antigen presented to them in the context of a major histocompatibility (MHC) class I molecule, in the case of CD8 T cells, or in the context of a MHC class II molecule, in the case of CD4 T cells.

In the mouse there are 3 different MHC class I molecules, referred to as H2K, D, L. Each of these molecules comes in many different allelic forms, each with clefts on its surface for the presentation of a wide variety of peptides predominantly generated from intracellular proteins (e.g. viral proteins synthesized in infected cells) by a process called antigen processing. There are motifs that govern the sequence of the various peptides

that a given MHC molecule may bind. For example, the H2D^b binding motif is characterized by a peptide sequence of 9-11 amino acids with two anchors: Asn at position 5 and an hydrophobic C-terminal residue (Met, Ile, Leu), while the H2K^b molecule binds octomers that have an aromatic amino acid at position 5 and a hydrophobic amino acid at position 8 (Falk et al., 1991).

Activation of naïve T cells requires two signals. Signal one is received through the TCR after engaging its cognate peptide-MHC complex on an APC (Schwartz, 1985). The affinity of TCR for MHC/peptide complexes is relatively low, being in the order of 10^{-5} to 10^{-7} M (Corr et al., 1994). Studies with CD4 T cells have shown that a threshold of approximately 8,000 TCRs must be triggered before T cell activation takes place (Viola and Lanzavecchia, 1996). However, the second costimulatory signal provided by one or more distinct APC surface molecules (e.g. B7.1/B7.2 and ICOS) interacting with coreceptors on the T cells (CD28 and ICOSL) (Sharpe and Freeman, 2002) can lower the activation threshold to 1,500 TCRs (Viola and Lanzavecchia, 1996). Signals mediated through CD40L-CD40 and CD137 (4-1BB)-CD137L interactions can further enhance interactions between a T cell and an APC (Grewal and Flavell, 1998; Halstead et al., 2002). It has been shown that a single peptide/HLA-DR complex is capable of triggering up to 200 TCRs in a serial fashion (Valitutti et al., 1995), and that a peptide only needs to occupy less than 0.08% of the total 258,000 H2D^b molecules present on the surface of a cell in order to stimulate a T cell (Christinck et al., 1991). At the immunological synapse, the rapidly formed area of contact between a T cell and an APC, membrane molecules are organized in supramolecular clusters with short molecules, such as TCR-CD3- ζ complex, CD2 and CD28, in a central region and large molecules, such as LFA-1

and CD45, in a peripheral ring. TCRs are continuously internalized and recycled. Those that are engaged by peptide-MHC are tethered into lipid raft microdomains, which contain kinases (Lck) and adapters (LAT), and initiate the signaling cascade (Cherukuri et al., 2001; Kane et al., 2000; Lanzavecchia and Sallusto, 2001). However, a recent report showing that TCR signaling precedes immunological synapse formation challenges current ideas about the role of immunological synapse in T cell activation (Lee et al., 2002). In addition, CD8 can act as a coreceptor, stabilizing the TCR/MHC-peptide complex and amplifying signals, or it can act as an adhesion molecule (Daniels et al., 2001). The second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, can increase intracellular Ca^{++} , leading to the activation of calcium-dependent kinases that then further phosphorylate other proteins, producing the changes in transcriptional regulation that result in T cell activation.

E. CD8 T cell response to viruses and its role in antiviral immunity and immunopathology during viral infections. Virus-specific CD8 T cell responses are important for the immunologic control of viral infections (Doherty et al., 1997; Gairin and Oldstone, 1992; Reddehase et al., 1987). The hierarchies of virus-derived CTL epitopes have been defined in numerous viral systems. In C57BL/6 mice, four H2D^b- and three H2K^b-restricted CTL epitopes have been defined for LCMV; five are contained within the glycoprotein (GP33-41, GP34-43, GP92-101, GP118-125 and GP276-286) and two are within the nucleoprotein (NP396-404 and NP205-212; Table 1) (Gairin et al., 1995; Hudrisier et al., 1997; van der Most et al., 1998; Whitton et al., 1988). The NP396, GP33, GP34 and GP276 epitopes are considered immunodominant, whereas NP205, GP118 and GP92 are subdominant. In B6 mice, two H2D^b-restricted and two

Table 1. Some known epitopes aligned in mice

Virus	Epitope	Sequence										MHC I restriction
LCMV	NP396-404	F	Q	P	Q	<u>N</u>	G	Q	F	<u>I</u>		D ^b
	GP33-41	K	A	V	Y	<u>N</u>	F	A	T	C		D ^b
	GP276-286	S	G	V	E	<u>N</u>	P	G	G	Y	C <u>L</u>	D ^b
	GP92-101	C	S	A	N	<u>N</u>	S	H	H	Y	<u>I</u>	D ^b
	GP34-43	A	V	Y	N	<u>E</u>	A	T	<u>C</u>	G	I	K ^b
	NP205-212	Y	T	V	K	<u>Y</u>	P	N	<u>L</u>			K ^b
	GP118-125	I	S	H	N	<u>E</u>	C	N	<u>L</u>			K ^b
Flu APR8	NP366-374	A	S	N	E	<u>N</u>	M	E	T	<u>M</u>		D ^b
	PA224-233	S	S	L	E	<u>N</u>	F	R	A	Y	<u>V</u>	D ^b
	NS2 ₁₁₄₋₁₂₁	R	T	F	S	<u>E</u>	Q	L	<u>I</u>			K ^b
	M1 ₁₂₈₋₁₃₅	M	G	L	I	<u>Y</u>	N	R	<u>M</u>			K ^b
MCMV	IE1pp89 168-176	Y	P	H	F	M	P	T	N	L		L ^d
	m04 pp34 243-251	Y	G	P	S	L	Y	R	R	F		D ^d
	M84 pp65 297-305	A	Y	A	G	L	F	T	P	L		K ^d
	m164 257-265	A	G	P	P	R	Y	S	R	I		K ^d

References: see page 9 and 11.

Underlined amino acid residues: D^b or K^b binding motifs

H2K^b-restricted CTL epitopes have been defined for influenza A pr/8/34, contained within the nucleoprotein (NP366-374), the polymerase (PA224-233), the nonstructural protein (NS2₁₁₄₋₁₂₁) and the matrix protein (M1₁₂₈₋₁₃₅, Table 1) (Belz et al., 2000; Flynn et al., 1998; Townsend et al., 1985; Vitiello et al., 1996). Four H2^d-restricted MCMV-specific CD8 T cell epitopes have been defined in Balb/c mice (Table 1) (Holtappels et al., 2000b; Holtappels et al., 2002; Holtappels et al., 2000c; Reddehase, 2000; Reddehase et al., 1989), but no VV-specific CD8 T cell epitope has been fully characterized. However, our laboratory has found that three VV peptides, which could be potential CD8 T cell epitopes, stimulate CD8 T cells from VV-infected mice to produce IFN- γ (Figure 23, and Selin et al., unpublished data).

The dissection of CD8 T cell-mediated immunity has greatly been facilitated due to the recent development of advanced techniques for staining antigen-specific CD8⁺ T cells with MHC tetramers (Altman et al., 1996) and dimeric MHC-IgG chimeras (Dal Porto et al., 1993), or examining the functionality of individual T cells with peptide-induced intracellular cytokine stains and ELISPOT assays (Doherty and Christensen, 2000; Openshaw et al., 1995). Consistent with previous studies demonstrating minimal virus-induced bystander expansion of T cell populations (Zarozinski and Welsh, 1997), these new approaches have shown that the majority of the proliferating CD8 T cells elicited by LCMV or influenza A virus infection are virus-specific (Butz and Bevan, 1998; Flynn et al., 1998; Murali-Krishna et al., 1998; Selin et al., 1999). The CD8 T cell response to a virus is divided into three segments, beginning with the activation and proliferation of CD8 T cells, continuing with the effector phase, and ending with the silencing of the response (Selin et al., 2000; Welsh et al., 1997). Recent studies suggest

that the proliferation and differentiation program is predetermined and can be initiated by only brief contact with the APC (Kaech and Ahmed, 2001; van Stipdonk et al., 2001). Due to independence of the magnitude of expansion, dose and duration of infection or amount of antigen displayed, the onset and kinetics of CD8 T cell contraction may also be programmed early after infection (Badovinac et al., 2002). Following the expansion of T cells, the repertoire of virus-specific T cells is significantly altered, and the altered profile is maintained in the memory T cell repertoire (Bousso and Kourilsky, 1999; Lin et al., 2000; Lin and Welsh, 1998). After the priming of virus-specific T cells within secondary lymphoid organs (spleen and draining lymph nodes), T cells express chemokine receptors that induce them to leave the lymphoid tissue and migrate to peripheral infection sites, where inflammatory chemokines are being synthesized (Price et al., 1999; Sallusto et al., 2000). It has been shown that activated T cells, once recruited, are mostly retained as nondividing cells in nonlymphoid tissues (Harris et al., 2002). However, a small population (10%) of Ag-specific CD8⁺ T cells is maintained in the lung airways by homeostatic proliferation and migration to lung airways from the lymphoid organs after viral clearance (Hogan et al., 2002).

The potent CD8 T cell response can not only efficiently clear virus but also induce substantial immunopathology. LCMV induces a potent CD8 T cell response, in which at least 40% to 60% of the CD8 population is LCMV-specific (Murali-Krishna et al., 1998), leading to clearance of virus. LCMV persists in mice devoid of CD8⁺ T lymphocytes as a result of disruption of the beta 2-microglobulin gene (Lehmann-Grube et al., 1993). The same T cells responsible for viral clearance can mediate a severe leptomeningitis if the virus is replicating in the brain (Doherty and Zinkernagel, 1974;

Zinkernagel and Doherty, 1975).

In influenza A virus infection of mice, 20% of the CD8 population in the bronchoalveolar lavage is influenza A virus NP366-specific (Flynn et al., 1998). CD8 T cells play a critical role in recovery from murine influenza A virus infection (Yap et al., 1979), and β 2-M knock-out mice that lack MHC class I-restricted T cells have delayed viral clearance and increased mortality after influenza A virus challenge (Bender et al., 1992). However, in experimental murine influenza A virus infection, immunodeficient mice were observed to have a more protracted time to death than immunocompetent mice after lethal intranasal (i.n.) inoculation, indicating immune-mediated immunopathology (Bot et al., 1996; Scherle et al., 1992; Wells et al., 1983). Virus-specific CD8+ cytotoxic T cells have also been shown to contribute to pathologic manifestations of influenza A virus infection in a TcR transgenic mouse model (Moskophidis and Kioussis, 1998). The cellular targets of influenza A virus-specific CTLs may participate directly in the development of progressive tissue injury by becoming activated in response to interaction with the T cells and producing proinflammatory mediators (Small et al., 2001).

During VV infection, antiviral cytokines such as IFN- γ produced by activated CD8 T cells are important to clear VV (Ruby and Ramshaw, 1991). In this thesis, there will be more evidence that activated CD8 T cells also contributed to immunopathology in the mouse lung in the late stages of VV infection. MCMV-specific CD8 T cells play a critical role in controlling MCMV infection (Holtappels et al., 1998; Holtappels et al., 2001; Quinnan et al., 1978; Steffens et al., 1998). However, in a model of bone marrow transplantation, once a critical MCMV load was reached, CD8 T cells produced IFN- γ ,

which then stimulated macrophages to make high levels of $\text{TNF-}\alpha$, leading to aggravation of direct CMV-associated cytopathic effects (Barry et al., 2000). Therefore, the CD8 T cell response that efficiently clears virus may sometimes induce substantial immunopathology.

F. Memory CD8 T cells and their role in antiviral immunity and immunopathology during viral infections. Upon secondary infection, the faster memory T-cell response, in association with antibody responses, can control infections quickly and eliminate the pathogen (Kaeche et al., 2002). This rapid memory T cell response occurs due to a substantial increase (~ 1000 -fold) in the precursor frequency of antigen-specific T cells in immune animals (Flynn et al., 1998; Murali-Krishna et al., 1998; Selin et al., 1996). Memory CD8 T cells are also at a higher activation state than naïve T cells (Pihlgren et al., 1996; Tabi et al., 1988; Veiga-Fernandes et al., 2000). Expression of increased levels of cytokine receptors and adhesion molecules on memory CD8 T cells further enhances their ease of activation (Dutton et al., 1998; Razvi et al., 1995). The gene-expression profile of memory T cells is reprogrammed by changes in chromatin structure and the profile of active transcription factors. For example, the genes that encode $\text{IFN-}\gamma$ and cytotoxic molecules, such as perforin and granzyme B, are constitutively expressed in effector and memory CD8^+ T cells (Bachmann et al., 1999; Grayson et al., 2001; Masopust et al., 2001; Selin and Welsh, 1997; Yang et al., 1998). Although the synthesis of these proteins occurs in an 'on-off' fashion that is regulated by antigen contact, elevated levels of the mRNA transcripts endow memory CD8^+ T cells with the capacity to produce larger quantities of these proteins more rapidly than naïve T cells (Kaeche and Ahmed, 2001; Veiga-Fernandes et al., 2000). Memory CD8^+ T cells

express a different pattern of surface proteins that are involved in cell adhesion (CD62L) and chemotaxis (CCR7) from naive T cells, allowing memory T cells to extravasate into non-lymphoid tissues and mucosal sites (Moser and Loetscher, 2001). This enables memory T cells to survey peripheral tissues, where microbial infections are generally initiated. Recently, memory CD8⁺ T cells that reside in these peripheral tissues have been termed 'effector' memory T cells, whereas those that are found in lymphoid organs have been termed 'central' memory T cells (Masopust et al., 2001; Sallusto et al., 1999; Weninger et al., 2001). Memory CD8 T-cell populations are maintained for a long time (Homann et al., 2001; Selin et al., 1996) due to slowly turning over at a slow, yet steady, pace (Razvi et al., 1995; Sprent et al., 1999) in a process called homeostatic cell proliferation. Cytokines such as IL-2, IL-15, IL-7, and IFN α/β might make important contributions to this homeostatic process, but their roles have yet to be fully defined (Ku et al., 2000; McNally et al., 2001; Schluns et al., 2000; Tan et al., 2002; Zhang et al., 1998). Recently, it was shown that memory T cells could use either IL-7R α - or IL-15-mediated signals for acute homeostatic proliferation in a lymphopenic host and that basal homeostatic proliferation of memory CD8 T cells in a full T cell compartment was blocked completely in IL-15-deficient hosts (Goldrath et al., 2002). In contrast to CD8⁺ T-cell memory, a recent study indicated that virus-specific CD4⁺ T-cell memory decreases slowly over time (Homann et al., 2001). It is interesting to speculate that the differential stability of CD4⁺ and CD8⁺ T-cell memory could stem from the distinct effects of IL-15 on the respective populations — memory CD8⁺ T cells proliferate in response to IL-15, whereas memory CD4⁺ T cells do not (Tan et al., 2002; Zhang et al., 1998). Therefore, it is the increased number of antigen-specific T cells, and their faster

responses, anatomical location (that is, near the sites of microbial entry) and longevity that collectively explain how memory CD8 T cells confer long-term protective immunity.

Although memory CD8 T cells can not act like neutralizing antibodies to prevent infection from being initiated, memory T cells do prevent the infection from progressing and protect against a lethal viral challenge. Evidence that memory cells keep a persistent infection from progressing is that enrichment of IE1 pp89 peptide-specific CD8 T cells was observed in a pulmonary memory CD8 T cell pool during latent MCMV infection of the lungs (Holtappels et al., 2000a). In the case of secondary infections with influenza A virus or LCMV, the recall response and inflammatory process in the infection sites develop more rapidly and to a much greater level, thus the virus is eliminated 2 to 3 days earlier than a primary infection (Belz et al., 2000; Welsh et al., 1997). There also is a risk of enhanced immunopathology with a greater secondary response, especially with a secondary infection after vaccination that induces CD8 T cells but no protective antibodies. For instance, in DBA/2 mice which were previously vaccinated with either a major or minor LCMV T-cell epitope-containing vaccine, subsequent LCMV infection aggravated disease and caused lethal immunopathology (Oehen et al., 1991). T cell-mediated enhanced immunopathology was also observed in mice immunized with DNA encoding an internal LCMV virion protein (Zarozinski et al., 1995). This thesis will provide insight into memory T cell-mediated immunopathology during heterologous viral infections.

G. T cell cross-reactivity and its possible role in the activation of memory CD8 T cells. Much work has been done to examine cross-reactivity between viruses and viral proteins. It is clear that similarities in linear sequences of amino acids

can lead to cross-reactive immunity (Mathew et al., 1998). However, a TCR that recognizes a given MHC-presented peptide might also recognize diverse peptides that have the appropriate MHC binding motif and have, projecting from the antigen-binding groove, amino-acid side chains that are able to stimulate the TCR. As a result, T cell cross-reactivity may differ from antibody cross-reactivity and be much less predictable based simply on amino acid sequence similarity. This T-cell degeneracy was first uncovered by unexpected cross-reactivity in that VSV- and influenza A virus-specific CTL clones were shown to cross-react with uninfected allogeneic targets (Braciale et al., 1981; Sheil et al., 1987). Cross-reactive epitopes may exist within a virus or a single viral protein. The RSV M2 protein contains two distinct cross-reactive epitopes that share very little amino acid similarity outside of the anchor residues required for presentation by H2K^d (Kulkarni et al., 1993). An influenza A virus NP-specific clone was shown to lyse targets that were coated with an unrelated peptide derived from a different influenza A virus-encoded protein PB2 (Anderson et al., 1992), and an H2K^d-restricted CTL clone was also shown to recognize two distinct and very different peptides from the HA and NS1 proteins of influenza A virus (Kuwano et al., 1991). Cross-reactivity has also been documented to occur between viral and self-proteins. A K^d-restricted CD8 T cell line specific for influenza A virus HA was found to cross-react with a peptide derived from the Ig heavy-chain variable region (Cao et al., 1995). HSV-1 specific T cells were shown to cross-react with corneal autoantigens and cause stromal keratitis, and these T cells also cross-reacted with a peptide derived from IgG2a (Avery et al., 1995). Studies of several virus infections in mice and of EBV infections in humans have shown that a high degree of allospecific CTL activity is generated during infection (Burrows et al., 1994;

Yang and Welsh, 1986). This was first attributed to non-specific, polyclonal bystander activation, but limiting-dilution clonal assays have shown that much of this activity can be attributed to T-cell clones that are cross-reactive between virus-infected syngeneic targets and uninfected targets that express allogeneic MHC antigens (Burrows et al., 1999; Nahill and Welsh, 1993). Two structural studies that examined T-cell cross-reactivity against allogeneic cells have shown that the same TCR can bind to different peptide-ligand structures (Daniel et al., 1998; Speir et al., 1998).

Cross-reactivity has also been demonstrated between different viruses. An influenza A virus matrix-protein-specific clone was shown to cross-react with a rotavirus VP4 peptide (Shimojo et al., 1989). An immunodominant, HLA-A2-restricted T-cell epitope that is encoded by hepatitis C virus (HCV NS3-1073; CVNGVCWTV) has seven out of nine amino acids in common with an influenza A virus immunodominant peptide (NA-231; CVNGSCFTV), and T cells cross-react with the two epitopes (Wedemeyer et al., 2001).

Cross-reactivity may account for reactivation of memory T cells specific for one virus during heterologous virus infections. CTLs that are specific for LCMV were reactivated in LCMV-immune mice that were challenged with Pichinde virus (PV), VV or MCMV (Yang et al., 1989). Again, this was speculated originally to be due to the polyclonal bystander activation of memory CTLs, but clonal analyses showed T-cell clones cross-reactive between LCMV and PV, and between LCMV and VV (Selin et al., 1994). Based on positional analysis of various amino-acid substitutions at different residues of a peptide, it has been calculated that a given TCR has the potential to recognize a million different peptide-MHC combinations (Mason, 1998). This result

suggests that peptides do not necessarily need to have high sequence homology to be cross-reactive with the same T cell. Moreover, memory T cells are in a higher activation state than naïve cells, and they can be productively stimulated by a peptide concentration that is 50 times lower than that required for the stimulation of naïve T cells (Pihlgren et al., 1996; Tabi et al., 1988; Veiga-Fernandes et al., 2000). Therefore, it would not be surprising if a memory T cell could be stimulated by a cross-reactive peptide with substantially less affinity for the TCR than the original peptide that created the memory T-cell pool. Cross-reactivity between unrelated viruses could influence the CD8⁺ T-cell memory pool and viral pathogenesis, which is the very important aspect of memory T cell-mediated heterologous immunity.

H. Antigen-independent T cell stimulation by cytokines and its possible role in the activation of memory CD8 T cells. Functional activation of *naïve T cells* has been well known to require TCR-triggering and appropriate costimulatory signals. However, there are some misleading studies, which propose TCR-independent bystander activation of *naïve T cells*. LCMV-specific transgenic CD8 T cells were shown to acquire IL-2-dependent cytolytic effector function *in vivo* during acute infection with unrelated pathogens such as VV or *Listeria monocytogenes* (Ehl et al., 1997), but these experiments did not exclude the possibility of cross-reactivity between the transgenic T cells and the unrelated pathogens. Another study showed that treatment with IL-15, in the absence of exogenous antigen, led to cell division as well as an increase in the expression of the activation marker CD69 on naïve CD8 T cells *in vitro* (Kanegane and Tosato, 1996). However, a role for TCR stimulation in this observation was not excluded. Unlike naïve T cells, memory CD8⁺ T cells, which are at a higher

activation state than naïve T cells, are able to respond to stimuli in a bystander manner in the apparent absence of the cognate antigen (Tough et al., 1999).

Certain combinations of cytokines, including type I IFN and IL-15, have been shown to induce memory CD8 T cell activation and differentiation in the absence of the cognate antigen. CD8 T cells with a memory phenotype (CD44^{hi}) will incorporate the DNA precursor BrdU and upregulate CD25 after *in vivo* exposure to type I IFN (Tough et al., 1996; Tough et al., 1997). Memory CD8⁺ T cells have receptors for IL-15, a cytokine produced by monocytes and other cells. IL-15 has biological activities similar to those of IL-2, including growth stimulation of NK cells and activated T cells and induction of cytolytic function of NK cells. Moreover, IL-15 seems to regulate their homeostasis (Ku et al., 2000; Sprent et al., 1999). During viral infection, there is an induction of type I interferon, which can then induce IL-15 production in macrophages and dendritic cells. In turn, IL-15 can enhance the division of memory CD8⁺ T cells, as shown by the uptake of BrdU *in vivo* (Ku et al., 2000; McNally et al., 2001; Tough et al., 1996). This division does not lead to a significant increase in the total number of CD8⁺ T cells (McNally et al., 2001). In fact, homeostatic division might be necessary during the virus-induced IFN response because of type I IFN-induced apoptosis of memory CD8⁺ T cells (McNally et al., 2001). Homeostatic proliferation of memory CD8 T cells is largely MHC I independent (Tuma and Pamer, 2002), but TCR-dependence is yet unclear.

IL-12, IL-18 and IFN- γ can induce activation and homeostatic proliferation of memory CD8 T cells. IL-12 is another important cytokine for controlling many viral and mycobacterial infections. It regulates Th1/Th2 responses, promotes specific CTL responses, and enhances the lytic activity of NK cells. IL-12 also enhances the

production of IFN- γ from memory T cells and NK cells (Trinchieri G, 1995). IL-18 is a cytokine with potent IFN- γ -inducing activity and plays an important role in the T_H1-mediated immune response in collaboration with IL-12 (Akira, 2000). Recently, IL-12 has been reported to drive memory CD8 T cells into homeostatic cell division *in vivo* (Kieper et al., 2001) and this effect is IFN- γ or IL-18-dependent and possibly through IL-15 signaling (Tough et al., 2001). Bystander proliferation of memory CD8 T cells was also observed upon culture of memory CD8 T cells with NKT or T cells activated by superantigens. The proliferation could be induced by an IL-12- or IFN- γ -dependent but IFN- α/β -independent pathway (Eberl et al., 2000).

A few studies suggest that non-specifically stimulated T cells, which do not seem to increase substantially in number, have some antimicrobial effector functions like producing IFN- γ . One study showed that bystander activation of memory-phenotype CD8 T cells contributes to the rapid production of IFN- γ in response to bacterial pathogens, and this was dependent on IL-12 and IL-18 (Lertmemongkolkchai et al., 2001). GADD45 β is a protein that activates mitogen-activated protein kinase extracellular signal-regulated kinase kinase 4 (MEKK4) in T cells. This is required for cytokine-induced IFN- γ transcription. IL-18-stimulated GADD45 β was required in IL-12 and IL-18-induced but not TCR-induced IFN- γ production (Yang et al., 2001). However, IL-15 induced memory CD8 T cell proliferation but was unable to drive cytokine production in the absence of TCR activation or IL-12 stimulation *in vitro* (Niedbala et al., 2002).

It is difficult to exclude a role for TCR stimulation during the cytokine-induced activation of memory T cells, because all TCRs have some low level of reactivity against

endogenous ligands, the expression of which might be up-regulated by exogenous or virus-induced cytokines. Studies showed that transgenic T-cell populations that were not specific for the virus did not increase in number in the spleen during viral infections (McNally et al., 2001; Zarozinski and Welsh, 1997). This does not negate the possibility that the non-specific T cells experience some level of activation. Recent BrdU-labeling studies in influenza A virus- and mouse γ -herpesvirus-infected mice have indicated that antigen-specific T cells divide much more rapidly than bystander T cells (Belz and Doherty, 2001; Flynn et al., 1999; Turner et al., 2001). In a study that used two influenza A virus strains that encode closely related T-cell epitopes, there was a substantially greater proliferation of T cells that are cross-reactive with the challenge-virus epitope than of T cells that are specific only for the previously encountered virus (Haanen et al., 1999). Therefore, antigen-stimulated T cells have a greater potential in response to stimuli than bystander T cells.

During a viral infection, it is difficult to be certain when T cells are responding as a result of bystander mechanisms or cross-reactive stimulation with viral peptides. Despite the above observations a complete absence of TCR stimulation was seldom conclusively shown, and the physiological relevance of T cell activation by such high levels of diverse cytokines and its importance to viral infections are unclear.

I. Modulation of memory CD8 T cells during sequential viral infections. A memory T cell, which is easier to activate, would be sensitive to low affinity cross-reactive TCR-triggering. Moreover, memory T cells may also be much more susceptible to cytokine stimulation in the absence of cognate antigen. It is important to understand the functional characteristics of this complex population during sequential

viral infections, where both cross-reactive epitopes and bystander stimulating cytokines could be present. The previous studies conducted in our laboratory on heterologous immunity with systemic viral infections (Selin et al., 1994; Selin et al., 1998; Yang et al., 1985) laid the groundwork to my thesis research. However, the knowledge of modulation of memory CD8 T cells during sequential viral infections was still very limited. Among the studies conducted in our laboratory discussed below, some of those were done concurrently with the studies presented in this thesis.

Cross-reactivity between heterologous viruses alters T cell immunodominance hierarchies (Brehm et al., 2002). Carefully controlled experiments in mice have indicated that the hierarchy of the T-cell response to immunodominant peptides is consistent and predictable (van der Most et al., 1998; Yewdell and Bennink, 1999). Hence, the specificity of the response is similar between genetically identical animals. Despite this, the TCR usage differs from animal to animal (Lin and Welsh, 1998). Previous exposure to other pathogens can alter the hierarchy of the T-cell repertoire. LCMV and PV encode cross-reactive CD8⁺ T-cell epitopes (LCMV NP205 and PV NP205) that have six out of eight amino acids in common (Brehm et al., 2002). These peptides are subdominant in each infection; they elicit T-cell responses that account for less than 3% of the antigen-specific CD8⁺ T cells during acute infection and about 1% of the CD8⁺ T cells in the memory pool. If LCMV-immune mice are infected with PV, or if PV-immune mice are infected with LCMV, the T-cell responses to these NP205-specific peptides become dominant, involving more than 20% of the CD8⁺ T cells. Hence, infections with heterologous agents can affect immunodominance when cross-reactive peptides are present. This is similar to the concept of original antigenic sin that was used to describe

memory Ab responses and more recently to describe the cross-reactive T-cell responses to variants of the same viruses (Haanen et al., 1999; Klenerman and Zinkernagel, 1998). Original antigenic sin is an asymmetric pattern of antibody or CTL cross-reactivity determined by exposure to previously existing strains (De St Groth and Webster, 1966). This brings in to question any studies of immunodominance hierarchies in response to human viral infections, as we have no idea how previous infections may have influenced these hierarchies.

Following heterologous viral infections, there is attrition of memory CD8 T cells while cross-reactive T cells remain in the memory CD8 T cell populations. In the absence of antigenic stimulation, the high frequency of CD8⁺ memory T-cells is stable in a host that has previously experienced a viral infection. This can be disrupted by infection with heterologous viruses. After only 2 to 3 subsequent infections, there were 5- to 8-fold reductions in memory CD8⁺ T cells specific for previously encountered pathogens (Selin et al., 1999; Selin et al., 1996). One of the factors related to this loss in the long-term memory T-cell pool may be the type I IFN-dependent apoptosis of memory CD8⁺ T cells that are specific for agents other than the infecting virus (McNally et al., 2001). When there are cross-reactive epitopes between the heterologous viruses, cross-reactive T cell responses may enrich cross-reactive memory CD8 T cell populations. For example, T cells that recognize cross-reactive NP205 epitopes between LCMV and PV are preserved and might be enriched in the memory population in either LCMV- or PV-immune mice challenged with the heterologous virus (Brehm et al., 2002). Antigen is not required to maintain memory CD8⁺ T cells (Murali-Krishna et al., 1999), but cross-reactive antigen will offset the non-specific deletion of memory T cells (attrition) that occurs during new

infections.

Memory CD8 T cells specific for one virus respond differently to cytokines and to a heterologous virus infection. Examinations of adoptively transferred CFSE-labeled LCMV-immune donor T cells in Thy-1 congenic hosts inoculated with viruses or with the cytokine inducer poly I:C (Kim et al., 2002) found that poly I:C stimulation caused a limited synchronized division of memory CD8 T cells specific to each of five LCMV epitopes, with no increase and sometimes a loss in number, and no change in their epitope hierarchy. Infections with the heterologous viruses PV and VV caused more than seven divisions (similar to homologous LCMV challenge) and increases in number of T cells specific to some putatively cross-reactive but not other epitopes and resulted in substantial changes in the hierarchy of the LCMV-specific T cells. Thus, depending on the pathogen and the sequence of infections, a heterologous agent may selectively stimulate the memory pool.

Cross-reactive CD4⁺ T-cell responses between heterologous viruses have not been examined systematically. There is a gradual erosion of the memory CD4⁺ T-cell response with time (Homann et al., 2001). It is of interest, however, that heterologous viral infections do not accelerate the decline in CD4⁺ T-cell memory, perhaps because relatively few CD4⁺ T cells enter the memory pool and compete with the resident population (Varga et al., 2001).

J. T cell-mediated heterologous immunity and immunopathology.

The term "heterologous immunity" was coined to describe a phenomenon occurring between concomitant infections with different bacteria or intracellular parasites several decades ago (Clark, 2001). This type of heterologous immunity is thought to be mediated

by pro-inflammatory cytokines and nitric oxide produced by macrophages. Furthermore, some cases in which the protection that *Mycobacterium tuberculosis* bacillus Calmette-Guerin (BCG) gives against parasites or other bacteria has been shown to be fully active for many months after the initial BCG challenge and to be through a mechanism that is effective in the absence of antibody (Clark et al., 1977), suggesting that other components such as T cells through activating macrophages may play a role in this long-lasting heterologous immunity between bacterial and parasites. However, the steps along the pathway of antigen recognition are still imperfectly understood in those cases (Clark, 2001).

A recent study indicates that prior immunity to a virus will confer partial protection against unrelated viruses during systemic i.p. infections and this heterologous immunity is T cell-dependent (Selin et al., 1998). This heterologous protection was significant, although it was considerably less than the almost total protection that is seen after challenge with a homologous virus. Adoptive-transfer studies indicated that protection against PV and VV was mediated by a combination of CD4⁺ and CD8⁺ T cells from LCMV-immune mice.

A history of unrelated viral infections can also greatly influence immunopathology. LCMV-immune mice infected i.p. with VV clear VV more rapidly than naïve mice but also develop immunopathology in the form of acute fatty necrosis of their peritoneal fat pads (Selin et al., 1998; Yang et al., 1985). T cells have an important role in immunopathology, as the lesions in fat were dependent on CD8 and CD4 T cells from LCMV-immune mice (Selin et al., 1998).

The T_H1 or T_H2 bias of a memory response might affect the T_H1 or T_H2 bias of the primary response to a heterologous agent. In a murine model of respiratory syncytial virus (RSV) infection, if mice are infected with influenza A virus (inducing a T_H1 -type response) before the recombinant VV-RSV immunization [inducing a T_H2 -type response (Alwan et al., 1994)] and RSV challenge, the immune response to RSV is altered and the infection resolves quickly without a strong T_H2 response and serious eosinophilia (Walzl et al., 2000).

Heterologous virus infections can result in exacerbations and remissions of autoimmune conditions. Cross-reactive CD4 T cell responses between pathogens and self-antigens have been associated with autoimmune diseases. For instance, HSV-1 infections have been associated with the induction of T cell-dependent autoimmune reactions to corneal tissue (Zhao et al., 1998). In addition, infection of SJL/J mice with Theiler's murine encephalomyelitis virus (TMEV) leads to a late onset of CD4 T cell-mediated demyelinating disease similar to multiple sclerosis (Olson et al., 2001). The onset of the demyelinating disease can be accelerated by a TMEV variant encoding the MBP peptide or a bacterial mimic peptide that shared 6 out of 13 amino acids with the MBP peptide.

Taken together, heterologous immunity can result in protective immunity, altered immunopathology, and changes in the T_H1/T_H2 balance in future pathogen exposure as well as induction of autoimmune diseases. However, our understanding of mechanisms involved in mediating heterologous immunity is still in its infancy.

K. Mucosally transmitted respiratory viral infections and immune system in the lung. Most viruses enter hosts through the mucosa (Nathanson and K.L.,

1997). For respiratory mucosal viral infections, some viruses such as influenza A virus tend to replicate only in the superficial epithelial cells of the mucosal tract, while many other viruses classically invade through the mucosa, but cause disease as a consequence of systemic spread (Doherty and Ahmed, 1997). Like influenza A virus infection, as long as the viral antigens can be recruited into, processed and presented in the secondary lymphoid tissues such as local draining lymph nodes and spleen, immune responses primed from both mucosal and systemic lymphoid organs can participate in the fight against viruses. Of course, mucosal IgA as well as IgG antibodies and easily activated resident cellular immune components such as macrophages and memory T cells are the first line of defense against mucosal virus infections.

In addition to nonspecific viral defense mechanisms such as mucus and soluble factors (e.g. protease inhibitor α_1 -antitrypsin, and complement), a highly integrated mucosal immune system exists in the lower respiratory tract. The immune system in the lung is composed of draining lymph nodes including mediastinal lymph node (MLN) and bronchial lymph nodes, and bronchus-associated lymphoid tissue (BALT), as well as the leukocytes of the interstitium, the bronchoalveolar and intravascular spaces (Pabst, 1992; Pabst and Tschernig, 1995; Tschernig and Pabst, 2000). BALT, less well defined than gut-associated lymphoid tissue (GALT), was first described as the nodules of lymphoid tissue containing T cells, B cells, macrophages and dendritic cells in the branch points of an airway or between the bronchus and an artery (Bienenstock et al., 1973a; Bienenstock et al., 1973b). The frequency of BALT in species other than rabbits and rats is variable, and certain stimuli are essential for the development of BALT. Studies in humans and experimental animal models show that the numbers of lymphocytes in the

lung interstitium are comparable to those of the circulating blood pool in the lung, and these interstitial lymphocytes have a characteristic subset composition, size, distribution and cytokine production profile (Nelson et al., 1990; Pabst, 1990). The lung intravascular pool of lymphocytes is large, on a per gram basis nearly ten times larger than for the liver or kidney (Pabst, 1990). Lymphocytes and lymphoblasts from this pool differ from peripheral blood or splenic lymphocytes in their migration patterns (Binns et al., 1992). The lymphocyte influx into the bronchoalveolar space from the circulating pool can be seen in some conditions such as influenza A virus infection. These cells can leave the bronchoalveolar space by entering the lung parenchyma and reaching the draining lymph nodes via afferent lymphatic vessels (Pabst and Binns, 1995).

L. The respiratory heterologous viral infection of C57BL/6 mice, as a model system to study heterologous mucosal immunity. Since many viruses enter the host through the mucosa of the respiratory tract, it is important to examine the role of memory T cells in immunity and immunopathogenesis with heterologous viruses which are inoculated via the natural intranasal mucosal route of infection. Another advantage of the mucosal system is that we will be able to assess heterologous immune responses with the common human virus, influenza A virus.

The viruses used in this thesis are known to be capable of being transmitted via the respiratory route and to induce distinctive lung pathologies. Using this baseline knowledge I could then assess what effect heterologous immunity would have on lung pathology. LCMV, a relatively common human pathogen, with 4-15% of the general population reported to be serologically positive, is predominantly transmitted via the respiratory route in the natural environment (Lehmann-Grube, 1986; Marrie and Saron,

1998). In arenaviral hemorrhagic fever patients, interstitial pneumonitis and pulmonary edema are sometimes seen (Walker and Murphy, 1987). One study with LCMV infection of guinea pigs showed that those infected with the more virulent LCMV WE strain had pneumonitis (Martinez Peralta et al., 1990). VV has been used for vaccination against smallpox which in the environment can be transmitted via the respiratory mucosa (Fenner et al., 1988; Wehrle et al., 1970). VV has also been used in a murine pneumonia model (Tufariello et al., 1994). In this study with recombinant VV, pulmonary edema (at higher dose) and infiltration of inflammatory cells, including mononuclear, polymorphonuclear and eosinophilic leukocytes around bronchioles and arterioles, were observed in the intranasally infected Balb/c mouse lungs. Influenza A virus causes a highly contagious acute respiratory illness among humans. Acute influenza A virus infection in mice induces loss of epithelium, airway obliteration, peribronchiolar and perivascular accumulation with mixed PMN and MN cells, as well as mild BALT in the lung (Lemerrier et al., 1979; Mackenzie et al., 1989). As the lungs become more and more consolidated, there is epithelial bronchiolization (Loosli et al., 1975; Yap et al., 1979). Day 30 to one year after acute infection, the lungs still show a patchy MN interstitial pneumonia, deposition of collagen in the affected areas, and mild BALT (Jakab et al., 1983). Interstitial pneumonia is a severe organ manifestation of CMV disease in the immunocompromised host, in particular in recipients of bone marrow transplantation. MCMV infections have been used as models for the human disease. When large i.n. dosages of MCMV are administered to BRVS mice, a diffuse and interstitial pneumonitis develops, characterized by thickening of the alveolar walls due to infiltration with mononuclear cells and the presence of proteinaceous fluid in the air spaces (Jordan, 1978; Staczek, 1999). It is important to

examine the influence, if any, of prior exposure to one of these viruses on immunity and immunopathogenesis to a later respiratory infection with a heterologous virus.

M. Objective of the thesis. The goal of this thesis was to study virus-specific memory CD8 T cells and their role in heterologous immunity and immunopathology in the mouse lung with respiratory viral infections that are inoculated via the intranasal mucosal route of infection. I have also taken advantage of several viral systems to pose the following questions:

1. What is the effect of prior immunity to one virus (LCMV, MCMV or influenza) on immunity against and immunopathology induced by a second unrelated virus in the mouse lung during a respiratory infection?
2. How are the frequency and repertoire of LCMV-specific memory CD8 T cells modulated during a respiratory heterologous VV infection?
3. What are the mechanisms involved in activation of LCMV-specific memory CD8 T cells during heterologous VV infections?
4. What are the immunological and immunopathological consequences of modulation of memory CD8 T cells specific to LCMV during the heterologous VV infections?
5. What are the effector mechanisms involved in mediating those immunological and immunopathological consequences during the heterologous viral infections?
6. Are patterns of lung immunopathology in murine models of respiratory heterologous viral infections reflective of human diseases?

CHAPTER II

MATERIALS AND METHODS

A. Mice

Six-week old C57BL/6J (B6, H2^b) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 IL-12 knock-out mice (B6.129-IL12B) were bred and maintained under microisolator conditions at the University of Massachusetts Medical School Department of Animal Medicine. All mice used in this study were between 2 months to 2 years of age and maintained at the University of Massachusetts Medical School Department of Animal Medicine.

B. Viruses

The Armstrong or clone 13 strains of LCMV, an RNA virus in the Old World arenavirus family, were propagated in BHK21 baby hamster kidney cells (Selin et al., 1994). The WR strain of VV, a DNA virus in the poxvirus family, was propagated in L929 cells (Selin et al., 1994). MCMV, strain Smith, a DNA virus in the herpesvirus family, was extracted from salivary glands of infected weanling BALB/c mice (Bukowski et al., 1984). The mouse-adapted influenza virus A/PR/8/34 (H1N1), a RNA virus in the orthomyxovirus family, was grown in the allantoic fluid of 10-day old embryonated chicken eggs (SPAFAS, Preston, CT). This virus was a kind gift of Dr. Harriet Robinson, Emory University, Atlanta. For acute virus infections, metofane-anesthetized mice were challenged intranasally with 4×10^5 PFU of LCMV, 400 or 10^5 PFU of MCMV, or 70 or 200

PFU of influenza virus. Various VV doses (1×10^3 - 1×10^5 PFU) were used, as there was some variability of virulence among different VV stocks.

C. Cell lines

MC57G (H2D^b), a methylcholanthrene-induced fibroblast cell line from C57BL/6 mice, and Vero African green monkey kidney cells were cultured in Eagle's MEM (Gibco laboratories, Grand Island, NY). Highly NK-sensitive YAC-1 cells, derived from a Moloney leukemia virus-induced lymphoma in A/Sn mice, were maintained in RPMI 1640 medium (Gibco Laboratories). Madin-Darby canine kidney (MDCK) cells were grown in Eagle's MEM (Gibco laboratories). GT-KO cells are mouse embryonic fibroblast lines cultured from α -1-3-galactosyltransferase (GT)-deficient transgenic mice. They were cultivated as monolayers in Dulbecco modified Eagle medium (Gibco BRL, Grand Island, N.Y.) (Welsh et al., 1998). All cell lines were supplemented with 100 U/ml penicillin G, 100 μ g/ml streptomycin sulfate, 2 mM L-glutamine, 10mM HEPES, and 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS: Sigma, St. Louis, MO).

D. Infection protocol

For mice that were acutely infected with a single virus, metofane-anesthetized mice were intranasally inoculated with a sublethal dose of the virus. At various times after the inoculation, the spleen, lung, and /or MLN were harvested, homogenized, and titrated for virus plaques. The lungs were also harvested, fixed and sectioned for histological evaluation. In most experiments, metofane-anesthetized mice were immunized with a sublethal dose of one virus. After the rise and fall of the acute T cell

response and when the immune system had returned to homeostasis (usually 6 weeks or later), the mice were challenged with the second heterologous virus. At various times after the second virus infection, the spleen, lung, and /or MLN were harvested, homogenized, and titrated for virus plaques. The lungs were also harvested, fixed and sectioned for histological evaluation. The viruses used were either purified over sucrose gradients and diluted in PBS (VV) or were diluted in PBS (LCMV; MCMV; and influenza A virus, containing allantoic fluid, diluted in PBS supplement with 1% BSA). Control naïve mice were intranasally immunized with culture media sedimented like virus over a sucrose gradient. All mice used were healthy with no evidence of any underlying disease. The immune mice were always age-matched to the control mice and housed under exactly the same pathogen-free conditions for the same time period.

E. Synthetic peptides

Synthetic peptides were generated by either Genemed Synthesis (San Francisco, CA) or American Peptide Company (Sunnyvale, CA) and were purified with reverse phase-high pressure liquid chromatography to 90% purity. Final products were analyzed by mass spectroscopy. The different sources yielded consistent data for all peptides examined. The following synthetic peptides were used. LCMV-specific peptides: NP396-404 (FQPQNGQFI; D^b binding), GP33-41 (KAVYNFATC; D^b binding), GP34-43 (AVYNFATCGI; K^b binding) GP276-286 (SGVENPGGYCL; D^b binding), NP205-212 (YTVKYPNL; K^b binding), GP92-101 (CSANNSHHYI; D^b binding) and GP118-125 (ISHNFCNL; K^b binding). VV-specific peptides: VVp1 (YNSLYPNV), VVp10 (STLNFNNL), and VVp24 (AIVNYANL). Influenza A virus-specific peptides: NP366-374 (ASNENMETM;

D^b binding) and polymerase A (PA224-233, SSLENFRAYV; D^b binding). Ovalbumin peptide: OVA257-264 (SIINFEKL; K^b binding).

F. Plaque assay

LCMV and VV were titrated by plaque assays on Vero cell monolayers. MCMV and influenza A virus were titrated by plaque assays on GT-KO and MDCK cell monolayers respectively. Individual tissues taken from virus-infected mice were put in 1 ml complete RPMI, homogenized, and stored at -80°C. The frozen aliquots were thawed on the day of the plaque assay and used to titrate the virus on cells grown in 6-well plates (Costar, Cambridge, MA). Serial ten-fold dilutions of 100 µl were added to the cells in 1 ml of MEM media and incubated for 90 min at 37°C with repeated rocking. A 1:1 mixture of 0.8% Seakemp agarose (FMC, Rockland, ME) and EMEM (BioWhittaker, Walkersville, MD) supplemented with antibiotics and 10% FCS was overlaid onto the cells. The plates were incubated at 37°C and on day 4 (LCMV) or 2 (VV) were stained with 2 ml of the above overlay mixture containing 0.1% neutral red (Sigma). Plaques were counted on the next day. For MCMV plaque assay, on day 5, the overlay agarose was gently removed from each well without damage of monolayers. Cells were then stained with 0.1% crystal violet (Sigma)-PBS containing 1% of paraformaldehyde for 10 min, and then rinsed once with PBS. The plates were air-dried, and plaques were counted. Virus titers were reported as log₁₀ PFU per whole organ. Results were expressed as the geometric mean titers per whole organ, i.e., the arithmetic average of the log₁₀ for 4 or 5 mice titrated for virus individually (±SEM).

For influenza A virus plaque assays, confluent MDCK cell monolayers were rinsed once with serum-free MEM and infected with 10-fold dilutions of samples in a total volume of 0.2 ml of PBS-0.2% BSA for 1 h with rocking every 5 to 10 min. Cells were then covered with an overlay of MEM containing 0.4% agarose, 0.2% glucose (Sigma), 0.002% DEAE-dextran, 0.01mg/ml Trypsin type III (Sigma), and 3x MEM vitamin solution (Gibco BRL). Cells were incubated at 37°C, and plaques were stained 3 days postinfection with 2 ml of the above overlay mixture containing 0.1% neutral red (Sigma). Plaques were counted on the next day.

G. Spleen and lymph node cell preparation

Spleens and lymph nodes were isolated and ground between the frosted ends of two glass microscope slides. The cell suspension was then passed through a fine nylon mesh to obtain a single cell suspension. Erythrocytes were lysed by briefly suspending the cell pellets in a 0.84% NH_4Cl solution before rinsing twice with RPMI 1640.

H. Isolation of lung lymphocytes

Lung lymphocytes were isolated using an adaptation of a previously described protocol (Khalil and Greenberg, 1989). The lung vascular bed was first flushed with 10 ml chilled HBSS introduced via cannulation of the right ventricle of the heart, with the aorta being first severed above the liver. Washing was performed until the lungs glistened clear white, at which point they were immediately excised and cleared of trachea, major bronchi, and all associated lymph nodes. The tissue was then minced into very small pieces and incubated for 1 h in 5 ml of HBSS (10% FCS) containing 125

U/ml collagenase I (Bibco, BRL), 60 U/ml DNase I (Sigma, St. Louis, MO), and 60 U/ml hyaluronidase (Sigma, St. Louis, MO). To obtain a single cell suspension the incubation media and tissue were passed through a 1-mm mesh to remove undigested tissue. The resulting cell suspension was layered over Lympholyte-M (Cedarlane Labs., Hornby, Ontario, Canada) and centrifuged at 500 g for 20 min at room temperature. The resulting mononuclear band was collected, washed, and analyzed.

I. Limiting dilution assay (LDA)

C57BL/6 mice were inoculated intranasally with LCMV, and at the indicated times after infection spleens were harvested. The LCMV-specific precursor frequency (p/f) per CD8⁺ cell was quantified by LDAs of unsorted splenocytes. The percentage of CD8⁺ T cells was determined by fluorescent antibody staining and FACS[®] analysis, as described below. For the LDAs, splenic lymphocytes from infected mice were harvested and titrated in U-bottomed, 96-well plates with 24 replicates at each dilution. They were stimulated with virus-infected peritoneal cells ($3-4 \times 10^4$ /well) and supplemented with irradiated splenic feeders ($1-2 \times 10^5$ /well) and growth factors provided by using a 16% culture supernatant from IL-2-secreting, gibbon lymphoma tumor cell line MLA.144 (American Type Culture Collection, Rockville, MD). After 4 d, the cultures were fed with 10^4 irradiated, virus-infected peritoneal cells. On days 7-8 of culture, individual wells were split twofold and assayed for cytolytic function on LCMV-infected or uninfected syngeneic target cells (KO) using a modified ⁵¹Cr-release assay. ⁵¹Cr-labeled targets (5×10^3) were added to all wells, and after 8-10 h incubation at 37°C the supernatant was harvested. Positive wells were defined as those wells whose ⁵¹Cr-release exceeded the mean

spontaneous release by >3 standard deviations. All wells that lysed uninfected syngeneic targets were eliminated from the analysis. Frequencies were calculated using χ^2 analysis (Taswell, 1981) on a computer program kindly provided by Dr. Richard Miller (University of Michigan, Ann Arbor, MI).

J. Flow cytometry

For multicolor FACS analysis, approximately 1×10^6 cells were stained in staining buffer (1% FCS and 0.02% NaN_3 in PBS). Stained cells were fixed with 2% paraformaldehyde, washed and resuspended in staining buffer and analyzed on either a FACS[®] 440 (Becton-Dickinson, San Jose, CA) or FACSstar[®] plus (Becton-Dickinson). Single color controls were used in all multiparameter FACS analyses for electronic compensation settings on the flow cytometer. Data analysis was performed using the program CELL QUEST (Becton-Dickinson).

K. Peptide-induced intracellular IFN- γ staining

Isolated leukocytes from the spleens, lungs or MLNs from the pooled mice were stained for intracellular IFN- γ based on the protocol from Cytofix/Cytoperm Plug[™] with Golgiplug[™] Kit (Pharmlngen, San Diego, CA). The isolated cells were stimulated with individual peptides (5 $\mu\text{g}/\text{ml}$) in the presence of 10 U/ml human recombinant IL-2 and 0.2 μl Golgiplug[™] (1% Brefeldin A in 100%DMSO) for 5 h at 37°C. The cells were then blocked by purified 2.4G2 antibody (Pharmlngen) specific for Fc receptors for 15 min at 4°C and stained with FITC-conjugated anti-mouse CD8 (clone 53-6.7) and CD69 PE (clone H1.2F3, Pharmlngen). Cells were fixed and permeabilized with Cytofix/Cytoperm

Plug™ solution (4% formaldehyde; 0.1% saponin) for 20 min at 4°C. They were then washed with 1X perm/wash™ solution (0.009% Sodium azide; 0.1% saponin) before adding PE or APC-conjugated rat anti-mouse anti-IFN- γ mAb (clone R4-6A2, Pharmingen) or control PE or APC-conjugated rat IgG1 isotype. All stainings with the isotype control were consistently <0.04%. Cells were analyzed on either a FACS® 440 (Becton Dickinson) or FACSstar® plus (Becton Dickinson). The total number of CD8+ or the total number of CD8+ cells secreting IFN- γ was determined by multiplying the percentage times the total cell yield from each mouse. For each experiment, lymphocytes from 3 mice were pooled in order to sample a large number of mice in experiments which involved very large numbers of samples where at the same time responses to 5 peptides plus isotype controls at 4 different time points in 3 organs were examined. Also, it was difficult to obtain the number of lymphocytes needed to test 5 peptides from the lung or MLN of one mouse.

L. H2D^b and H2K^b-IgG1 MHC dimer staining and modified intracellular staining of *in vivo* IFN- γ

Lymphocytes immediately after isolation from mouse lungs were stained with a mouse D^b/K^b-IgG1 fusion protein conjugated with β 2-microglobulin and the appropriate peptide (Greten et al., 1998; Selin et al., 1999). Briefly, 1 μ g of the peptide-D^b/K^b-IgG1 MHC dimer mixture was incubated with cells for 1.5 hr at 4°C. The cells were then stained with FITC-conjugated anti-CD8 (clone 53-6.7) and biotinylated anti-mouse IgG1 (Pharmingen) for 30 min at 4°C, followed by incubation with streptavidin PE (Pharmingen) for 20 min at 4°C. The cells were then fixed and permeabilized with

Cytofix/Cytoperm PlugTM solution. They were stained with APC-conjugated anti-IFN γ mAb (clone R4-6A2, Pharmingen) or control APC-conjugated IgG1 isotype. As no *in vitro* stimulation or incubation was carried out, any positive staining for intracellular IFN- γ indicated that the CD8 T cells producing IFN- γ would have been activated "*in vivo*" directly by the infection. All stainings with the isotype control were consistently <0.04%. A second control was also done using purified anti-IFN- γ mAb (clone R4-6A2, Pharmingen), which specifically blocked intracellular IFN- γ staining with APC-conjugated anti-IFN- γ mAb. The cells were analyzed either on a FACS[®] 440 or FACSstar[®] plus (Becton Dickinson).

M. NP205-specific H2K^b-IgG1 MHC dimer or H2K^b MHC tetramer staining and peptide-induced intracellular IFN- γ staining

Isolated cells were stimulated with individual peptides (5 μ g/ml) in the presence of 10 U/ml human recombinant IL-2 and 0.2 μ l GolgiplugTM (1% Brefeldin A in 100% DMSO) for 5 h at 37°C. For dimer staining, the cells were then blocked by purified 2.4G2 antibody (1 μ l/well, Pharmingen) specific for Fc receptors for 15 min at 4°C. Then 1 μ g of NP205-212 peptide-D^b/K^b-IgG1 MHC dimer mixture was incubated with cells for 1.5 hr at 4°C. The cells were then stained with FITC-conjugated anti-CD8 (clone 53-6.7) and biotinylated anti-mouse IgG1 (Pharmingen) for 30 min at 4°C, followed by incubation with streptavidin PE (Pharmingen) for 20 min at 4°C. For tetramer staining, the cells were blocked by purified 2.4G2 antibody (Pharmingen) specific for Fc receptors and streptavidin at 20 μ g/ml for 15 min at 4°C. After washing, the cells were resuspended in

100 μ l staining buffer containing PE-conjugated NP205-212-specific tetramer at 1 mg/ml and FITC-conjugated anti-CD8 (clone 53-6.7) for 1 hr at 4°C.

After cell surface staining, the cells were fixed and permeabilized with Cytofix/Cytoperm Plug™ solution (4% formaldehyde; 0.1% saponin) for 20 min at 4°C. They were then washed with 1X perm/wash™ solution (0.009% Sodium azide; 0.1% saponin) before adding APC-conjugated rat anti-mouse anti-IFN- γ mAb (clone R4-6A2, Pharmingen) or control APC-conjugated rat IgG1 isotype. Cells were analyzed on either a FACS® 440 (Becton Dickinson) or FACSstar® plus (Becton Dickinson).

N. Direct ex vivo cytotoxicity assay

Cell-mediated cytotoxicity was determined using a standard ^{51}Cr -release CTL assay (Selin and Welsh, 1997). MC57G target cells were infected with LCMV and VV at a multiplicity of infection (MOI) of 0.1 and 10 PFU/cell, and incubated for 2 d and 2h at 37°C respectively. YAC-1, MC57G and virus-infected MC57G cells were pelleted and resuspended in 100 μCi of Na^{51}Cr (Amersham Corp., Arlington Heights, IL) per 10^6 cells and incubated at 37°C in a humidified 5% CO_2 incubator for 1 h. Two-fold serial-diluted effector lung leukocytes or splenocytes were plated in triplicates to achieve the desired E:T ratio. ^{51}Cr -labeled target cells (5×10^3), either uninfected or infected with LCMV or VV, were added to all wells. For a spontaneous ^{51}Cr release control, 0.1 ml of complete MEM or RPMI 1640 was substituted for effectors. Maximum release was determined by adding 0.1 ml of 1% Nonidet P-40 to the well (United States Biochemical, Cleveland, OH). After a 6 h incubation at 37°C, the plates were centrifuged at 200 X g for 5 min, and 25 μ l of supernatant was harvested from each well and counted on a gamma

counter (Model 5000; Beckman instruments, Inc, Palo Alto, CA). Results were presented as percent specific ^{51}Cr -release = $100 \times [(\text{experimental cpm} - \text{spontaneous release cpm})/(\text{maximum release cpm} - \text{spontaneous release cpm})]$. The spontaneous release for each target used in these assays was < 20%.

O. Cytokine RNase protection assay

Total RNA was extracted from the whole lung tissue or isolated leukocytes from the spleen, lung or MLNs using Trizol[®] agent (Gibco, BRL). Briefly, lung tissue samples from individual mice were homogenized in 1 ml of TRIZOL Reagent using a power homogenizer. For cell samples, $5-10 \times 10^6$ cells were lysed in 1 ml of TRIZOL Reagent by repetitive pipetting. The homogenized samples were incubated for 5 min at room temperature prior to adding 0.2 ml of chloroform (Mallinckrodt Baker, Inc., Paris, KY) per 1 ml of TRIZOL Reagent. The samples were shaken vigorously and centrifuged at no more than $12,000 \times g$ for 15 min at 2 to 8°C after incubation for 2 to 3 min at room temperature. The upper aqueous phase was transferred to a fresh tube, and the RNA was precipitated by mixing with 0.5 ml of isopropyl alcohol (Fisher Scientific, Fair Lawn, NJ) per 1 ml of TRIZOL Reagent used for the initial homogenization. After incubation for 10 min at room temperature, the samples were centrifuged at $12,000 \times g$ for 10 min at 2 to 8°C. The RNA pellet was washed with 75% ethanol, completely air-dried and dissolved in RNase-free water.

Detection and quantification of a variety of murine cytokine mRNAs were accomplished with the mutiprobe RNase protection assay system (Pharmingen). Briefly, a mixture of [γ - ^{32}P] UTP-labeled antisense riboprobes was generated from a panel of

different cytokine templates using an *in vitro* transcription kit containing GACU pool, DTT, RNasin, T7 RNA polymerase, RNase-free DNase, and transcription buffer. Each template set also contained templates for the murine housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32 (a murine ribosomal protein), to ensure equal loading of total RNA onto the gel. A predetermined amount of total RNA (20-40 μ g) was hybridized overnight at 56°C with 32 P-labelled antisense riboprobe mixture and then the samples were digested with RNase. RNase-protected RNA fragments were purified by ethanol precipitation, and then the samples were resolved on a 5% PAGE. The protected bands were observed after exposure of the gels to a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) and X-ray films (Kodak). The specific cytokine bands were identified on the basis of their individual migration patterns in comparison with the undigested probes. The bands were quantified by densitometric analysis with ImageQuant densitometric software (Molecular Dynamics).

P. Cytokine ELISA

Cytokine levels in sera or bronchial alveolar lavage (BAL) of mice were measured by enzyme-linked immunosorbant assay (ELISA) based on the protocol provided by Pharmingen. Briefly, an ELISA plate was coated by incubation overnight at 4°C with purified anti-cytokine capture monoclonal antibody diluted in 50 μ l/well PBS (1-4 μ g/ml). The plates were then washed with PBS-0.05% Tween 20 after each of the following steps. The coated plate was then blocked by 200 μ l blocking buffer containing 10% fetal bovine serum for 2 hr at room temperature. Samples (sera and BAL) were added at 100

μ l per well, and a standard curve was constructed for each plate by using eight 2-fold serial dilutions of recombinant mouse cytokine (Pharmingen). The plates were incubated overnight at 4°C followed by addition of the diluted (1 μ g/ml) biotinylated anti-cytokine detecting antibody at 100 μ l per well. After a 1-hr incubation at room temperature, 100 μ l of streptavidin-HRP (1/400 dilution in PBS-10% FCS; obtained from Sigma) per well were added, and the plates were incubated at room temperature for 30 min. After washing, plates were developed with 3,3',5,5'-tetramethyl-benzidine dihydrochloride peroxidase (Sigma) dissolved in 0.05 M phosphate citrate buffer, pH 5.0. The reaction was stopped with 25 μ l of 2 N H₂SO₄. Plates were read at 450 nm using a microplate reader (Emax). A standard curve for each assay was generated and cytokine levels were calculated by a software program Softmax@pro3.0 (Molecular Devices Corporation, Menlo Park, CA). The following pairs of mAb were used: anti-IFN- γ , R4-6A2 and biotinylated XMG1.2; anti-IL-6, MP5-20F3 and biotinylated MP5-32C11; anti-TNF- α , G281-2626 and biotinylated MP6-XT3; anti-IL-4, 11B11 and biotinylated BVD6-24G2 (all from Pharmingen). Limits of detection for IFN- γ , IL-6, TNF- α , IL-4 were 0.2 ng/ml, 0.4 ng/ml, 0.2 ng/ml, and 0.03 ng/ml, respectively.

Q. Depletion of NK cells *in vivo*

Mice acutely infected with VV were injected i.p. with 100 μ l (200 μ g) of purified anti NK1.1 mAb (clone PK136) at day 0 and 4 postinfection. This greatly reduced NK cell activity for at least an 8 day time period.

R. *In vivo* IFN- γ depletion

In vivo depletion of IFN- γ was performed by i.v. (day 0 and 3) and i.p. (daily) injection of 100 μ l of an ascites of rat anti-IFN- γ mAb (R4-6A2) or a control rat IgG1 isotype (clone A110-1, Pharmingen) following VV challenge. At day 6.5 after VV infection, the lungs, MLNs and spleens were harvested and underwent virus titration and histological analysis.

S. Adoptive transfer

Naive C57BL/6 mice were injected i.v. with one spleen equivalent ($\sim 5-10 \times 10^7$ cells) of naive splenocytes or LCMV-immune splenocytes, which were prepared under sterile conditions and challenged i.n. with 3×10^4 PFU of VV. At 6.5 d after infection, lungs, MLNs and spleens were harvested, and titrated for VV.

T. Lung histological evaluation

At various times after mice were either acutely infected with a single virus, immunized with a single virus or immunized first with one virus and then acutely challenged with a second virus, lungs were harvested, fixed in 10% neutral buffered formaldehyde and paraffin embedded. Tissue sections (5 μ m) were then stained with hemotoxylin and eosin. Each section slide was concurrently examined microscopically by four persons, among whom two pathologists (Dr. Isabelle Joris and Dr. Armando Fraire, at the University of Massachusetts Medical School the Department of Pathology) were blinded in regards to treatments the mice had received and two other members were Dr. Liisa Selin and myself. Scores for lung pathology were graded by the two

pathologists. The level of lung diseases was scored based on the distribution and the severity of disease from 0/- to 3/+++ (0, within normal limit; 1/+, 1-9% involvement of the lung parenchyma; 2/++, 10-49% involvement of the lung parenchyma; 3/+++, $\geq 50\%$ involvement of the lung parenchyma). Histological pictures of representative lung sections were taken using Nikon microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY) and Spot[®] RT (Real time) slider "F" mount digital camera and Spot[®] RT software v. 3.0 (Diagnostic Instruments, Inc., Sterling Heights, MI) under the objectives of 4x, 10x and 40x magnifications. The digital picture files were organized using Adobe[®] Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA).

U. Immunohistochemistry staining

Lungs from the control and LCMV-immune mice were frozen in OCT compound (Miles Co., Eikhart, IN). Sections (5 μm) were fixed in acetone for 10 min, air-dried and then immersed in 3% H_2O_2 in methanol for 10 min at room temperature to block endogenous peroxidase activity. After rinsing, the sections were incubated with biotin-labeled mAbs to CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 (clone 53-5.8), or CD19 (Clone 1D3), (all from PharMingen) overnight at 4°C in a humidified chamber. Horse serum was used in staining buffer to inhibit background staining. Sections were then stained with streptavidin-HRP (1:25 dilution; Pharmingen) for 30 min at room temperature in a humidified chamber. Slides were stained with diaminobenzidine (DAB, Sigma) solution for 3-5 min at room temperature. The reaction was stopped by rinsing in PBS, and the sections were counterstained with hematoxylin.

V. In situ MHC-tetramer/dimer staining and confocal laser scanner microscopy (CLSM) of lung tissue sections

NP396-specific D^b tetramer was prepared using a previously described protocol (Mylin et al., 2000). Lungs from the control and LCMV-immune mice were frozen in OCT compound (Miles Co., Eikhart, IN). Sections (20 μ m) were cut, mounted on microscope slides and blocked by purified 2.4G2 antibody (PharMingen) specific for Fc receptors for 15 min at 4°C, and then incubated overnight at 4°C with FITC-labeled anti-CD8 mAb (clone 53-5.8, PharMingen, 6 μ g/ml) and PE-labeled NP396-specific MHC tetramer (20 μ g/ml) in PBS/10% normal mouse serum, followed by washing 3 times with PBS/10% normal mouse serum (Skinner et al., 2000). Sections were protected by The ProLongTM antifade kit (Molecular Probe, Eugene, OR) before CLSM. Green (FITC) and red (PE) signals were collected on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system. Images were taken using a 40X NA1.4 objective. Possible overlapping signals between the different fluorochromes were subtracted.

W. Statistical analysis

Data, where appropriate, were analyzed by Student's t-test for each individual experiment using either SigmaPlot software (SPSS Inc, Chicago, IL) or Microsoft's Excel software (Microsoft Corporation, Redmond, WA). Statistical significance was established at a p value of <0.05.

CHAPTER III

MODULATION OF LCMV-SPECIFIC MEMORY CD8 T CELLS AND THEIR POTENTIAL ROLE IN HETEROLOGOUS IMMUNITY AND IMMUNOPATHOLOGY IN THE LUNG AGAINST VV

Virus-specific memory T cells, generated after the host has been vaccinated against or infected with a virus, can prevent the infection with the homologous virus from progressing. The efficacy of memory T cells may be due to the fact that they persist at relatively high frequencies and are constitutively at a higher state of activation than naive T cells (Flynn et al., 1998; Hogan et al., 2001a; Masopust et al., 2001; Murali-Krishna et al., 1998; Selin et al., 1996). They can be induced into cell cycle by cytokines such as IFN α / β and IL-15 (Tough et al., 1999) and can be triggered by relatively low affinity interactions through their TCRs (Bradley et al., 1993; Tabi et al., 1988). Some apparently resting memory T cells can be cytolytically active (Selin and Welsh, 1997).

Each individual experiences many sequential infections that give rise to memory T cell pools of significant size and complexity. Analysis of experimental i.p. infections in mice has suggested that memory T cells generated in response to one virus can indeed alter the course of disease in response to unrelated viruses (Selin et al., 1998). Based on our observations on heterologous immunity, we would predict that each new infection will activate some of these memory T cells and modulate the original T cell repertoire, which is associated with alteration of the disease course. The following study develops these hypotheses.

I focused my studies on a respiratory mucosal model of heterologous virus infection. Developments in the identification of antigen-specific T cell responses (Altman et al., 1996; Dal Porto et al., 1993; Openshaw et al., 1995) enabled me to examine the extent of heterologous memory T cell participation in a peripheral organ, the lung, which is the initial site of virus replication, as well as in the conventional lymphoid compartments. By immunizing mice first with LCMV, which has several well defined epitopes, it allowed us to examine the ability of a heterologous virus to reactivate discrete LCMV epitope-specific memory T cell populations. I show here that an acute respiratory VV infection functionally activates memory CD8⁺ T cells specific to LCMV *in vivo*. VV infection resulted in selective expansion of LCMV-specific T cells and modulation of the LCMV-specific T cell repertoire in association with modification of the lung pathology and disease outcome.

A. Respiratory infection of C57BL/6 mice with LCMV or VV

In order to study the effect of prior immunity to LCMV on immunity against VV during a respiratory infection, I first examined the kinetics of viral clearance during a respiratory infection of naïve C57BL/6 mice with LCMV or VV (Table 2). When mice were infected with LCMV strain Armstrong, which I used in most of the experiments, comparable LCMV PFUs were detected in the lung, the draining MLN and the spleen at day 3 postinfection. Seven days after infection, LCMV titers in these three organs decreased. By day 16 postinfection, no LCMV was detectable in these organs. The kinetics of LCMV clearance was slightly delayed but still comparable to systemic i.p. LCMV infection (Selin et al., 2000). In some of the early experiments, I used the more

Table 2. Kinetics of viral clearance from normal C57BL/6 mice

Challenge virus	Days post infection	Virus Titer (\log_{10} PFU/organ) ^c		
		Spleen	MLN (pooled)	Lung
LCMV Armstrong (4) ^a	3	5.5 ± 0.3	5.0	5.3 ± 0.1
	7	2.4 ± 0.2	1.5	3.6 ± 0.1
	$\geq 16^b$	<1.0	<1.0	<1.0
LCMV clone 13 (4)	5	4.7 ± 0.02	ND	5.1 ± 0.1
	7	3.9 ± 0.3	ND	5.2 ± 0.3
	$\geq 14^b$	<1.0	ND	<1.0
Influenza virus (2)	5	<0.1	ND	6.3 ± 0.1
	7	<0.1	ND	4.8 ± 0.1
	$\geq 10^b$	<1.0	ND	<1.0
VV (2)	3	1.7 ± 0.4	ND	6.4 ± 0.01
	5	1.5 ± 0.3	ND	6.6 ± 0.2
	7	<1.0	ND	6.7 ± 0.1
	9	<1.0	ND	6.1 ± 0.2
	11	<1.0	ND	4.5 ± 1.1
	13	<1.0	ND	3.0 ± 0.3
	15	<1.0	ND	<1.0

The infection of mice was done as described in Materials and Methods. a, The number in parentheses represents the number of mice per treatment group. b, virus was also titrated in the organs of the immune-mice (at least 6 weeks after the acute infection). c, Data are presented as the mean titers in each group \pm SEM.

highly disseminating LCMV clone 13. Seven days after infection, LCMV clone 13 titers in the lung and spleen were more than $1.5 \log_{10}$ higher than LCMV strain Armstrong titers (Table 2). Nonetheless, no LCMV clone 13 was detectable in these two organs by day 14 postinfection. The exact day for complete LCMV clearance during the respiratory infections is not known because of the limited time points examined. However, both LCMV strain Armstrong and clone 13 were no longer recovered from the lungs and spleens of LCMV-immune mice (> 6 weeks after LCMV challenge) by plaque assays (Table 2). Therefore, a respiratory infection of C57BL/6 mice with the selected dose of LCMV could be used to study the effect of prior immunity to one virus on immunity against a second unrelated virus.

When naïve mice were infected with a sub-lethal dose of VV (2×10^3 PFU), VV titers in the lungs sampled in one of the two representative experiments showed a prolonged plateau from day 3 of i.n. challenge until day 9, and then a decline thereafter (Table 2). Therefore, C57BL/6 mice were very sensitive to respiratory VV infection. By day 15, VV could not be detected in the lung homogenates. In the spleen, there was a low level of VV replication, and VV was cleared by day 7 postinfection. These data are similar to one study showing a similar kinetics of pulmonary Cowpox virus replication in Balb/c mice after aerosol infection (Bray et al., 2000).

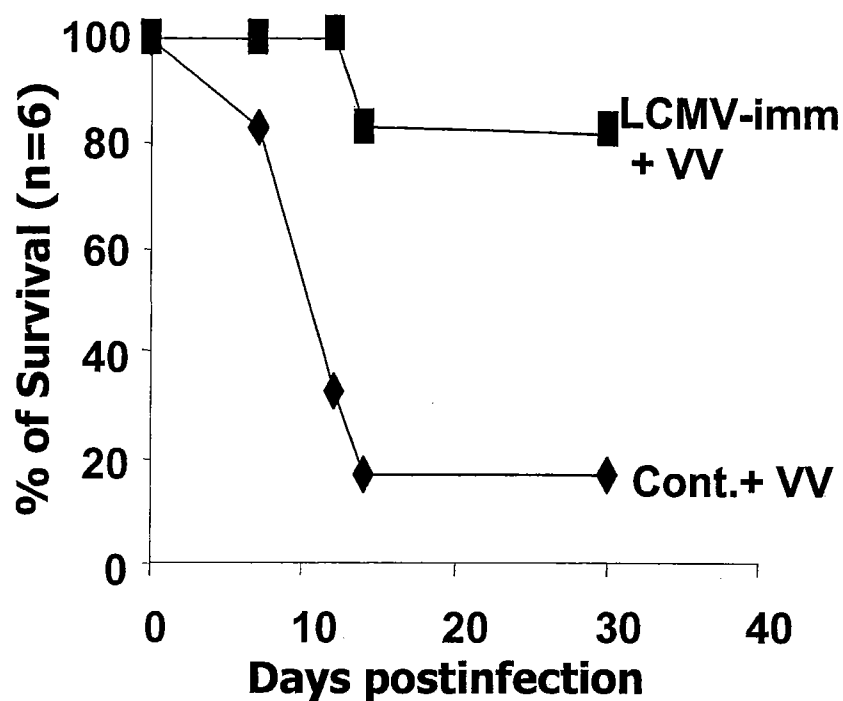


Figure 1. Decreased mortality of LCMV-immune mice challenged intranasally with a lethal dose of VV as compared to the age-matched control mice. Mice (n=6 mice/group) were infected with VV (1×10^4 PFU), and the time of their death after infection was recorded. These are representative data of 3 similar experiments.

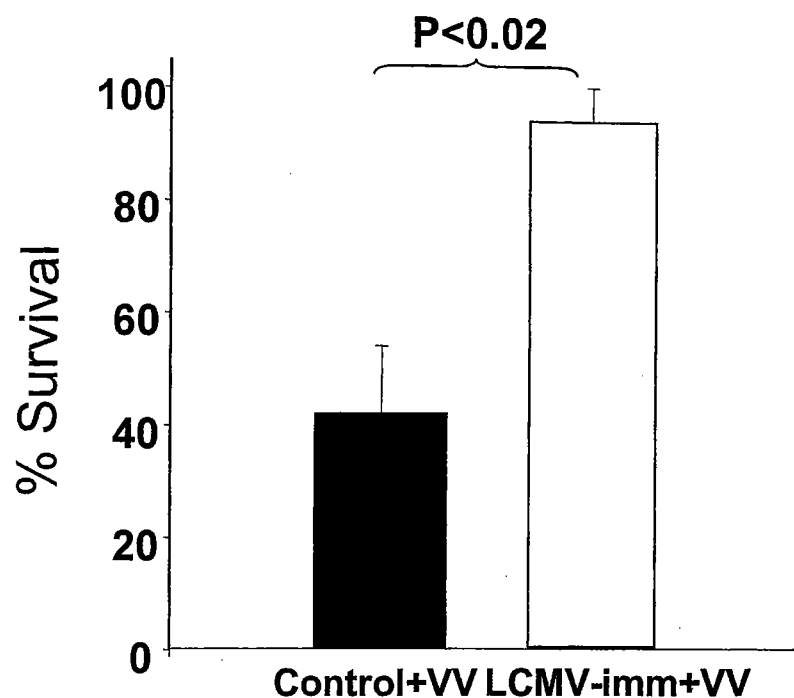


Figure 2. Immunity to LCMV provides protection against respiratory VV infection. Decreased mortality of LCMV-immune mice challenged with VV was seen as compared with the age-matched control mice. Data are presented as the mean \pm S.D. of % survival from 3 similar experiments, in which LCMV-immune mice were infected with VV **6, 12, and 18 weeks** after the initial LCMV infection, respectively.

B. Prior respiratory exposure to LCMV provides protection against a lethal VV challenge

When mice were infected with a lethal dose (1×10^4 PFU) of VV, there was, in the representative experiment shown in Figure 1, a marked difference between the groups: 83% of the LCMV-immune mice survived compared to only 17% of the control mice. Three similar experiments showed that the difference in mortality between the groups was statistically significant: $94 \pm 6\%$ of the LCMV-immune mice survived the lethal VV challenge compared to only $42 \pm 12\%$ of the control mice (Figure 2, $p < 0.02$). These experiments were carried out at different times from 6–18 weeks after LCMV infection (Figure 2), so this protection appeared to be independent of the time of VV challenge after LCMV infection. When the LCMV-immune and control mice were given a very high dose of VV (1×10^6 PFU), both groups of mice died, suggesting that the protection provided by heterologous immunity to LCMV was not at the same level as the protection conferred by homologous VV immunity. Nonetheless, the heterologous protection against a lethal dose of VV in the LCMV-immune mice was significant.

C. Time course of protective immunity to VV in LCMV-immune mice

Immune and control mice were acutely infected with a sub-lethal dose of VV, and the VV titers in the spleens and lungs were assessed at various time points (Table 3). By day 3 after infection there was a slight decrease in lung VV titers compared to that in LCMV-immune mice, but by day 5 there were lower VV titers in the spleens and lungs—95% ($P < 0.05$) and 97% ($P < 0.1$), respectively—of the LCMV-immune compared to the control mice. There continued to be a significant 83–90% ($P < 0.05$) reduction in

Table 3. Prior immunity to LCMV lowers VV titer during the course of a sub-lethal VV i.n. challenge

Immunizing virus	Days after VV challenge ^b	VV Titer (mean log ₁₀ PFU/organ \pm SEM) ^a	
		Spleen	Lung
Control	3	ND	6.1 \pm 0.3
LCMV		ND	5.9 \pm 0.5
Control	5	4.1 \pm 0.3	8.2 \pm 0.1
LCMV		<2.8 \pm 0.4 ^d	6.7 \pm 1.4 ^e
Control ^c	6	ND	6.0 \pm 0.03
LCMV ^c		ND	4.8 \pm 0.2 ^d
Control ^c	6	ND	6.2 \pm 0.3
LCMV ^c		ND	5.4 \pm 0.3 ^d
Control	7	<1.3 \pm 0	7.6 \pm 0.2
LCMV		<1.3 \pm 0	6.7 \pm 0.4 ^f

a, Lungs and spleens from VV-infected with mice were harvested and VV load was titrated. b, LCMV-immune mice were challenged with VV **10-15 weeks after initial LCMV infection**. 2-3 similar experiments were carried out at each time point. c, Mice acutely infected with VV were NK cell-depleted by i.p. injection of 100 μ l (200 μ g) of anti NK1.1 mAb (clone PK136) at day 0 and 4 PI. ND, not done. d, Statistically significant difference between the control and LCMV-immune mice ($P < 0.05$). e, $P = 0.1$. f, $P = 0.07$.

VV titers in the LCMV-immune mice lungs, as the VV infection progressed over days 6 and 7. Therefore, the decrease in mortality in the LCMV-immune mice was associated with a decreased VV load throughout the course of the acute VV infection.

Once again, the decrease in VV titer was independent of the time of VV challenge after LCMV infection (6 weeks after LCMV infection: control+VV, 5.6 ± 1.0 \log_{10} PFU/lung; LCMV-immune+VV, 6.5 ± 0.6 \log_{10} PFU/lung, Day 7 VV postinfection, $p < 0.1$, $n=4$ mice/group. 15 weeks after LCMV infection: Table 3). These differences in VV titers were also seen under more stringent conditions in which both groups of mice were depleted of NK cells, as indicated in the two experiments done on day 6 after VV infection (Table 3). These experiments suggested that NK cells did not play a role in this protection.

D. Adoptively transferred LCMV-immune splenocytes into naïve mice provide protection against VV challenge

It is predictable that residual effects of interferon and activated macrophages might provide a level of heterologous immunity immediately after infection. The heterologous immunity I have shown here is a long-lasting effect independent of the time of the initial immunization with one virus (Table 3). Moreover, as shown above, NK cells did not play a role in this protection. Since LCMV induces a potent T cell response, I hypothesized that the heterologous immunity against VV could be associated with prior immunity to LCMV.

To test this hypothesis, LCMV-immune splenocytes were adoptively transferred into naïve mice, which were then challenged with VV. As shown in Table 4, in the two

Table 4. Adoptively transferred LCMV-immune splenocytes into naïve mice provide protection against VV challenge

Exp #	Organ	Donor (n = number of recipients)	Virus Titer (mean log ₁₀ PFU/organ ± SEM)	Change (Immune v.s. Naïve)
No. 1	MLN (pooled)	Naïve-control (n = 6)	4.8	99%↓
		LCMV-immune (n = 7)	1.3	
	Lung	Naïve-control (n = 6)	6.5 ± 0.3	50%↓, P = 0.06
		LCMV-immune (n = 7)	6.2 ± 0.2	
No. 2	MLN (pooled)	Naïve-control (n = 3)	2.0	90%↓
		LCMV-immune (n = 4)	<1.0	
	Lung	Naïve-control (n = 3)	5.3 ± 0.3	80%↓, P = 0.3
		LCMV-immune (n = 4)	4.6 ± 0.5	

Naive C57BL/6 mice were injected i.v. with one spleen equivalent ($\sim 5-10 \times 10^7$ cells) of naive or LCMV-immune splenocytes, prepared under sterile conditions, then challenged i.n. with 3×10^4 PFU of VV. At 6.5 d after infection, lungs and MLNs were harvested and titrated for VV.

experiments there were 90% and 99%, respectively, reductions of VV titers in the pooled MLNs of mice adoptively reconstituted with LCMV-immune splenocytes at day 6.5 post VV infection as compared to naïve splenocytes. In the lungs, 50% reductions of VV titers were also observed in mice adoptively reconstituted with LCMV-immune splenocytes compared to naïve splenocytes. This suggested that the protective heterologous immunity against acute respiratory infection with VV was dependent on LCMV-immune splenocytes.

E. High frequencies of LCMV-specific memory CD8 T cells in lymphoid and peripheral tissues long after respiratory LCMV infection

To examine LCMV-specific memory CD8⁺ T cells before and during VV infection and to determine how they could be contributing to the protective immunity, I first defined the LCMV epitope-specific populations present in resting LCMV-immune mice. As determined originally by LDAs (Table 5), mice with respiratory LCMV infection developed high and stable frequencies of LCMV-specific memory CTL precursors (pCTL) in the spleen, equivalent to LCMV i.p. infection. There was no drop of the frequencies of pCTL from 2 (1/69 CD8 T cells) to 7.5 months (1/68 and 1/106) after LCMV infection.

The development of antigen-specific T cell staining using tetrameric (Altman et al., 1996) or dimeric (Greten et al., 1998) peptide/MHC complexes as well as intracellular cytokine staining (Openshaw et al., 1995) has made it possible to visualize antigen-specific T cells. Pooling of data on the intracellular IFN- γ responses to five individual epitopes (NP396, GP33, GP276, NP205, and GP92) showed a much higher percentage of LCMV-specific CD8⁺ T cells in the lung than in the MLNs (Figure 3a). The

Table 5. A respiratory LCMV infection induces a high frequency of CD8+ memory cells in mouse spleens, comparable to LCMV i.p. infection as determined by LDA^a

LCMV-immune	Frequency per total splenocyte	Range	% CD8+ splenocytes ^c	Frequency per CD8+ cell
i.p., 5 months^b	1/881	1/679-1/1254	13	1/88
# 1 (i.n., 2 months)	1/453	1/422-1/489	15	1/69
# 2 (i.n., 7.5 months)	1/611	1/553-1/683	11	1/68
# 3 (i.n., 7.5 months)	1/693	1/520-1/1035	15	1/106

a. LDAs were performed as described in Chapter II; b. the time after LCMV challenge; c, determined by FACS analysis.

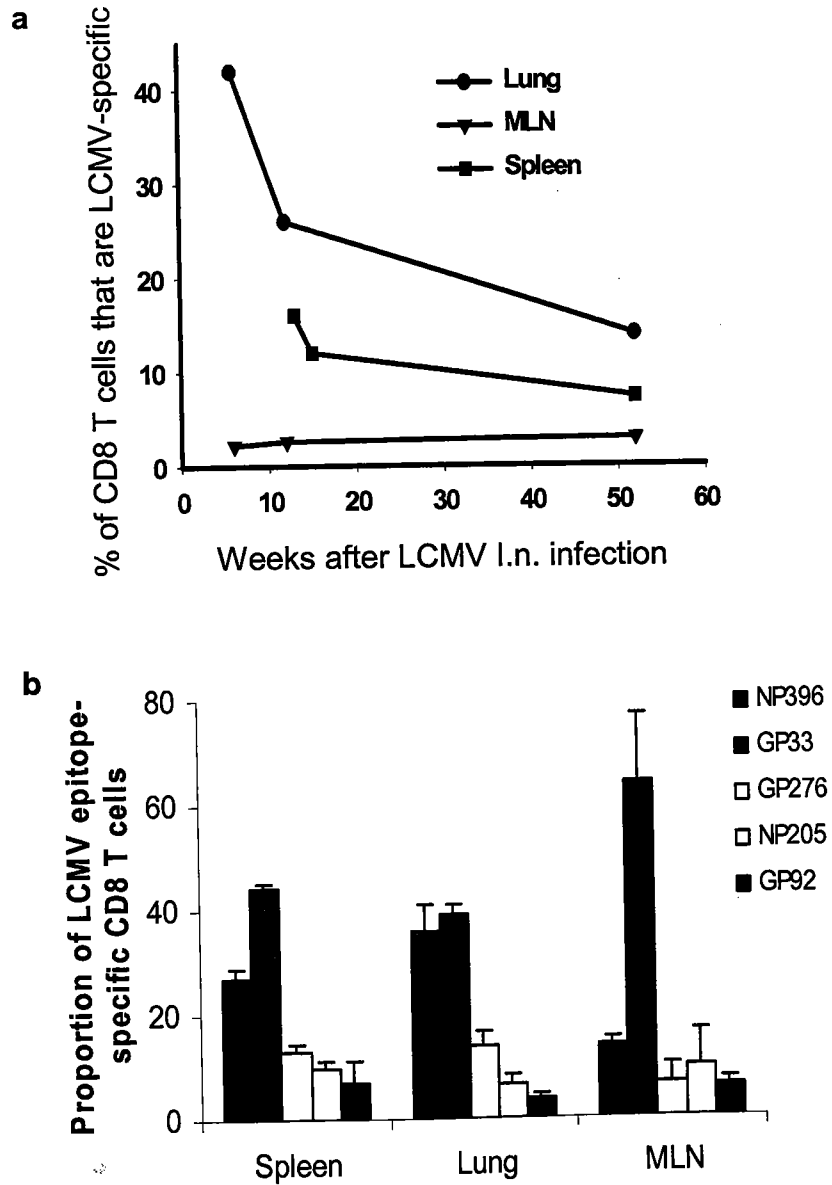
Figure 3

Figure 3. LCMV-specific memory CD8 T cells in resting LCMV-immune mice. Lymphocytes were isolated from the lungs, spleens and MLNs, stimulated with 5 individual peptides and stained for intracellular IFN- γ . Lymphocytes from groups of three mice each were pooled before staining. **a**, Y-axis represents the LCMV-specific CD8+ T cell frequencies. Data are representative of 2 similar experiments for lung and MLN, and 1 experiment for spleen. **b**. Different LCMV-specific repertoire in the MLN and lung of LCMV-immune mice. Data represent the mean of two (spleen) and three (lung and MLN) similar experiments. Cells were analyzed 6-13 weeks after LCMV infection for lung and MLN, and 13-15 weeks after LCMV infection for spleen.

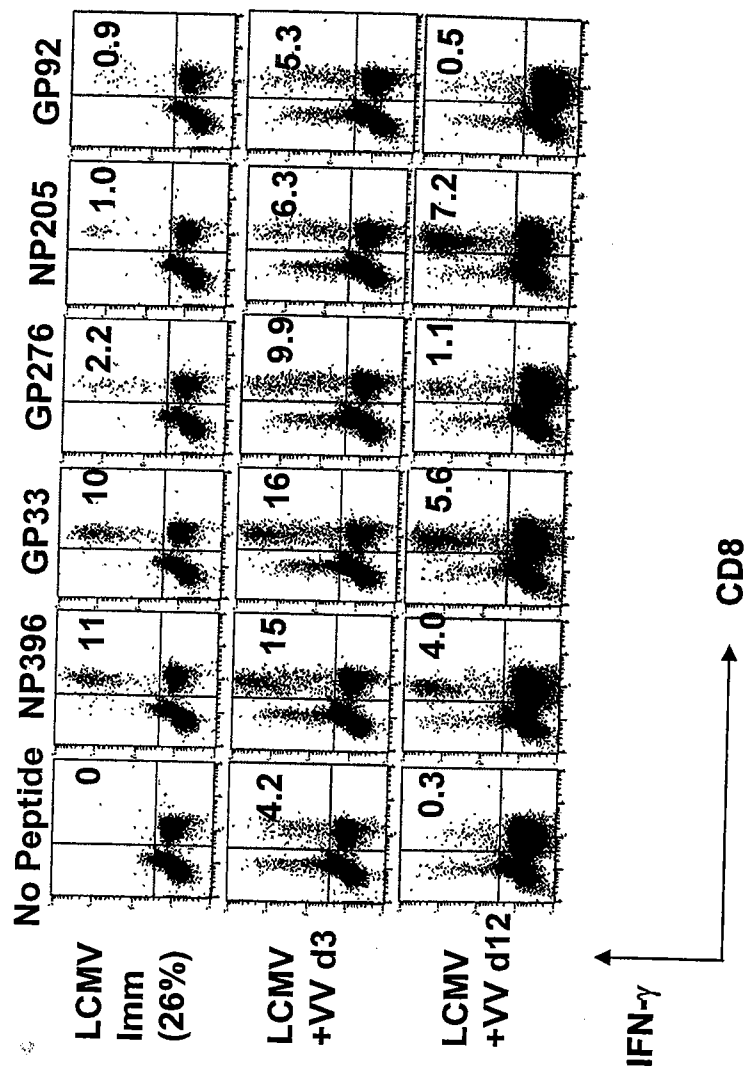
proportion of the CD8⁺ T cells that were LCMV-specific in the LCMV-immune lung decreased slowly from ~ 42% (2.5×10^5 cells) at 6 weeks to 26% (1.0×10^5 cells) at 12 weeks and to 14% (9.3×10^4 cells) at 52 weeks after i.n. LCMV infection. In contrast, the proportion of LCMV-specific CD8⁺ T cells in the MLNs of these same mice remained stable: 2.2% (2.0×10^3 cells) at 6 weeks; 1.6% (4.3×10^3 cells) at 12 weeks; and 2.0% (2.1×10^3 cells) at 52 weeks; $n = 3$ mice/group. The CD8⁺ memory T cell frequency in the spleen was higher than in the MLNs and also relatively stable after i.n. LCMV infection: 17% (2.0×10^6 cells) at 13 weeks; 13% (2.1×10^6 cells) at 15 weeks; 7.3% (1.6×10^6 cells) at 52 weeks; $n = 3$ mice/group. Observations about the stability of memory CD8⁺ T cells in the spleen after i.p. LCMV infection (Butz and Bevan, 1998; Murali-Krishna et al., 1998; Selin et al., 1999) and differences in the frequency of memory CD8⁺ T cells in lung, MLN and spleen were consistent with published data (Hogan et al., 2001a; Masopust et al., 2001).

Of interest was the unexpected result that the specificity of the CD8⁺ T cell response between MLN and lung differed. This was because up to 64% of the LCMV-specific CD8⁺ T cells in the MLN were GP33-specific, whereas T cells in the lung had broader specificities, comparably targeting NP396 (36%) and GP33 (39%) and with some recognition of GP276 and NP205 (Figure 3b). Analysis of the data from three similar experiments revealed 62% ($p=0.03$) and 53% ($p=0.08$) proportional reductions in NP396- and GP276-, specific CD8 T cells, respectively, in the MLN as compared to the lung. Meanwhile, there was 1.6-fold ($p=0.07$, $n= 3$ experiments) increase in the proportion of GP33-specific CD8 T cells in the MLN as compared to the lung. The alteration in proportions of these three immunodominant epitope-specific CD8 T cell

populations resulted in a different repertoire between the two organs in the LCMV-immune mice. The spleen tended to have a similar repertoire to the lung (Figure 3b). These results suggested that different CD8⁺ memory T cell homeostatic mechanisms were involved in the lung, its draining MLN, and the spleen.

F. Organ-specific accumulation of LCMV-specific memory CD8 T cells after VV challenge

After LCMV-immune mice were acutely challenged with VV, LCMV epitope-specific CD8⁺ memory T cells in both the lungs and MLNs were not diluted out, as one might have expected, by the VV-specific CD8⁺ T cell response (Figure 4 and 5). In fact, the total number of LCMV-specific CD8⁺ T cells in the MLN increased four-fold ($P < 0.05$) 3 days after VV challenge and continued to rise to six-fold ($P < 0.05$) higher by day 12 after infection (Figure 5). There was only a moderate increase in LCMV-specific CD8⁺ T cells in the lung 3 days after VV challenge, but by day 12 after infection there was a four-fold increase ($P < 0.1$, Figure 5). In contrast to these results, the spleen showed a significant 67% decrease ($P < 0.05$) of LCMV-specific CD8⁺ T cells by day 3 and a further decrease by day 7 ($P < 0.05$). Then, by day 12 after infection, the number of LCMV-specific CD8⁺ T cells returned to the numbers in resting LCMV-immune mice (Figure 5). All these changes occurred in the presence of VV antigens, as VV was still detectable in the lungs by day 12 post infection (Table 2). Therefore, there was an organ-specific accumulation of LCMV-specific memory CD8 T cells after VV challenge.

Figure 4**a. Lung**

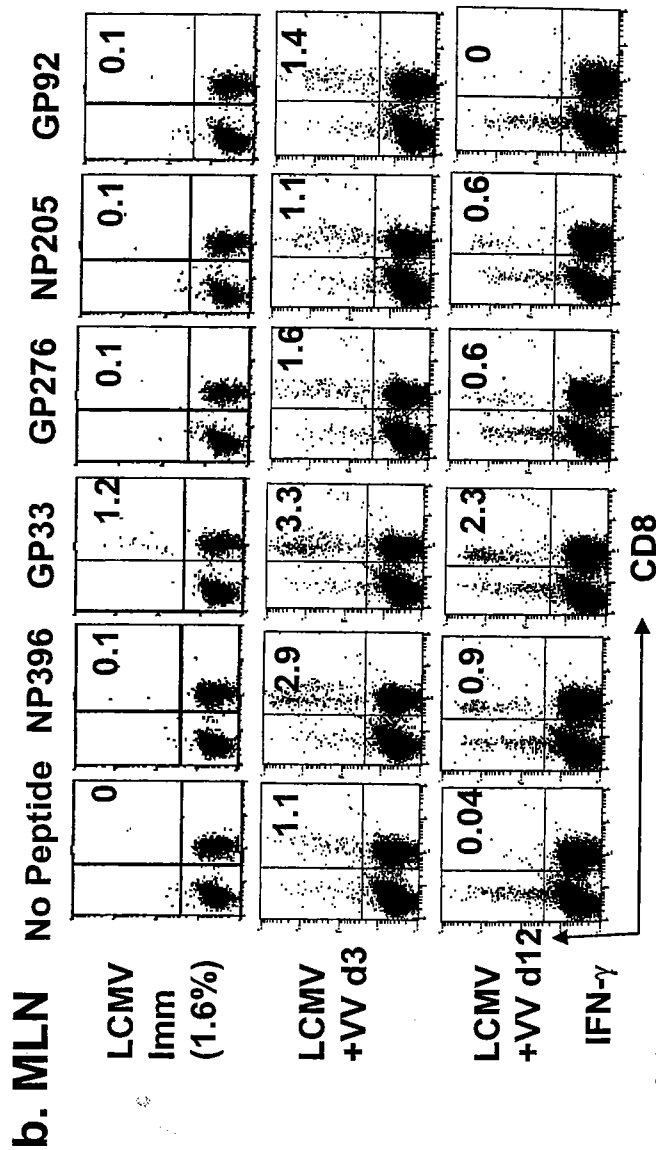


Figure 4. Persistence of LCMV epitope-specific memory CD8⁺ T cells in lungs and MLNs of resting LCMV-immune mice and during i.n. VV infection. LCMV-immune (>12 weeks after LCMV i.n. infection) and control mice were challenged i.n. with VV. Lymphocytes were isolated from the (a) lungs and (b) MLNs, stimulated with individual peptides and stained for intracellular IFN- γ . Lymphocytes from groups of three mice each were pooled before staining. The numbers in the right upper quadrants are the percentages of IFN- γ -staining CD8⁺ T cells specific for the indicated epitopes. The total LCMV-specific CD8⁺ T cell frequencies are given in parentheses. IFN- γ staining of cells from the VV-infected control mice was at background amounts (<0.2%). Data are representative of four similar experiments.

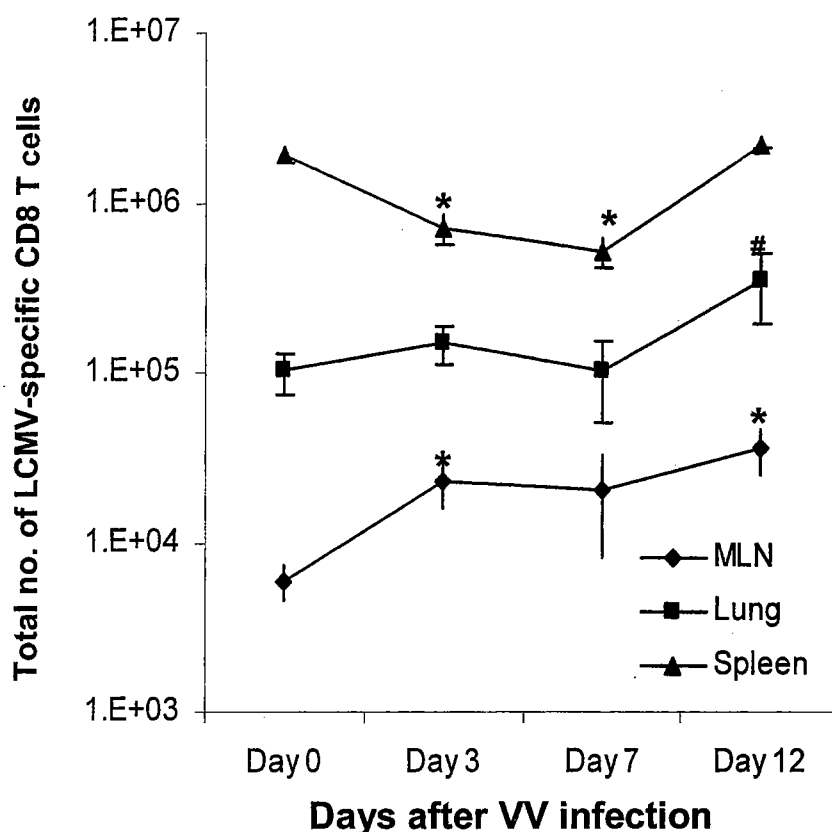


Figure 5. Organ-specific accumulation of LCMV-specific memory CD8 T cells after VV challenge. The total number of memory CD8⁺ T cells specific for each LCMV epitope was calculated by multiplying the total cell yield from each mouse organ by the percentage of IFN- γ -staining CD8⁺ cells specific for that epitope. The sum of the total numbers for each of five LCMV epitopes are shown. Because IFN- γ production by CD8⁺ T cells without peptide stimulation was observed, this was subtracted from each antigen-specific response before calculating the total numbers. Although some of these CD8⁺ T cells were LCMV-specific, we used conservative estimates. Statistically significant differences, between the unchallenged and the VV-challenged LCMV-immune mice, in the increase or decrease in total LCMV-specific CD8⁺ T cells, *, $p < 0.05$; #, $p < 0.1$. Data represent the mean of four similar experiments. For each experiment, lymphocytes from groups of three mice each were pooled before staining.

G. Repertoire alteration in LCMV-specific memory CD8 T cells in organ-specific manner after VV challenge

To examine whether there was a VV-induced nonspecific bystander equal accumulation of all LCMV-specific CD8⁺ T cells in the lungs and MLNs or whether there was some selectivity to this accumulation, I analyzed the proportion of each epitope-specific CD8⁺ T cell population in the total LCMV-specific pool (Figure 6). As already noted, there was a different LCMV-specific repertoire in the resting LCMV-immune MLN and lung: GP33-specific CD8⁺ T cells dominated the repertoire in the MLN, whereas NP396- and GP33-specific CD8⁺ T cells co-dominated in the lung. We found that VV infection modified the LCMV-specific repertoire differently in both these two organs in such a manner that it further enhanced the difference between these two sites. In the MLN, by day 12 after VV, there was a significant ($P < 0.05$) and consistent skewing of the LCMV repertoire in all four experiments, which resulted in increased proportions of NP396- and GP276-specific CD8⁺ T cells and a decreased proportion of GP33-specific CD8⁺ T cells, respectively (Figure 6). In the lung there was a different skewing of the LCMV-specific repertoire, and, although between experiments there was more variability in the lung than in the MLN, there was always a marked change in the selective specificities, with either the GP33- or NP205-specific responses dominating the repertoire. In one representative experiment (Figure 6), there was a seven-fold selective expansion of the NP205-specific population, retention of the GP33-specific population, but a 50–67% loss of the NP396- and GP276-specific populations. Overall, these data indicated that VV infection in an LCMV-immune mouse can markedly alter, in an organ-dependent manner, the LCMV-specific memory T cell population.

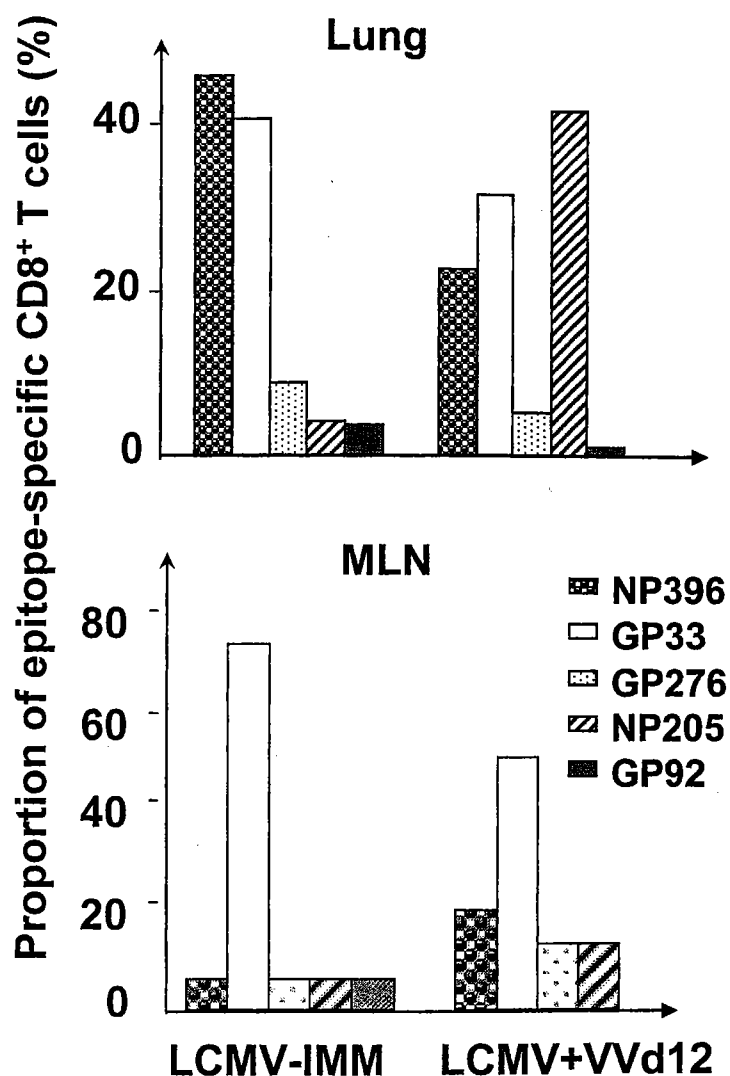


Figure 6. Alterations in the LCMV-specific CD8⁺ T cell repertoire in the VV-infected LCMV-immune mouse MLN and lung. Data were derived from intracellular IFN- γ staining analysis in Figure 10 a and b with five known LCMV-specific epitopes and are representative of four similar experiments. LCMV-IMM, resting LCMV-immune mice; LCMV+VVd12, LCMV-immune mice 12 days after-VV infection.

H. Up-regulation of early activation marker CD69 on LCMV-specific CD8 T cells in the VV-infected LCMV-immune mice

To examine if there was any evidence that these accumulating LCMV-specific memory CD8 T cells were activated during VV infection, I stimulated T cells with peptide *in vitro* to identify LCMV-specific T cells and then co-stained those cells with mAb to the early activation antigen CD69 (Figure 7). Just 3 days after VV infection, there were dramatic increases in percents of LCMV epitope-specific CD8⁺ CD69⁺ memory T cell populations in the lung and MLN of LCMV-immune mice. By day-12 PI, the percentages of LCMV epitope-specific CD8⁺ CD69⁺ memory T cell populations returned to the same levels as before VV infection, even though IFN γ was still induced. Figure 7 only shows CD69 staining patterns on NP396 and GP33-specific CD8 T cells. Three other tested epitope-specific CD8 T cell populations (GP276, NP205 and GP92) showed similar patterns for CD69 surface staining (not shown). Although peptides induced CD69 expression on CD8 T cells in some circumstances, these results would suggest that the LCMV-specific memory CD8 T cells were at a higher activation state early after VV infection than in a resting LCMV-immune mouse or on day 12 PI.

To directly visualize *in vivo* CD69 expression on LCMV epitope-specific CD8 T cells after VV challenge, I performed the CD69 staining without any *in vitro* stimulation and combined this with epitope-specific MHC dimer staining on ice. As shown in Table 6, 3 days after VV infection, there were dramatic 3 to 10-fold increases in percents of CD69⁺ NP396⁺, GP33⁺, and NP205-specific CD8 memory T cell populations in the lung of LCMV-immune mice. Following *in vitro* peptide stimulation all five tested LCMV-epitope-specific

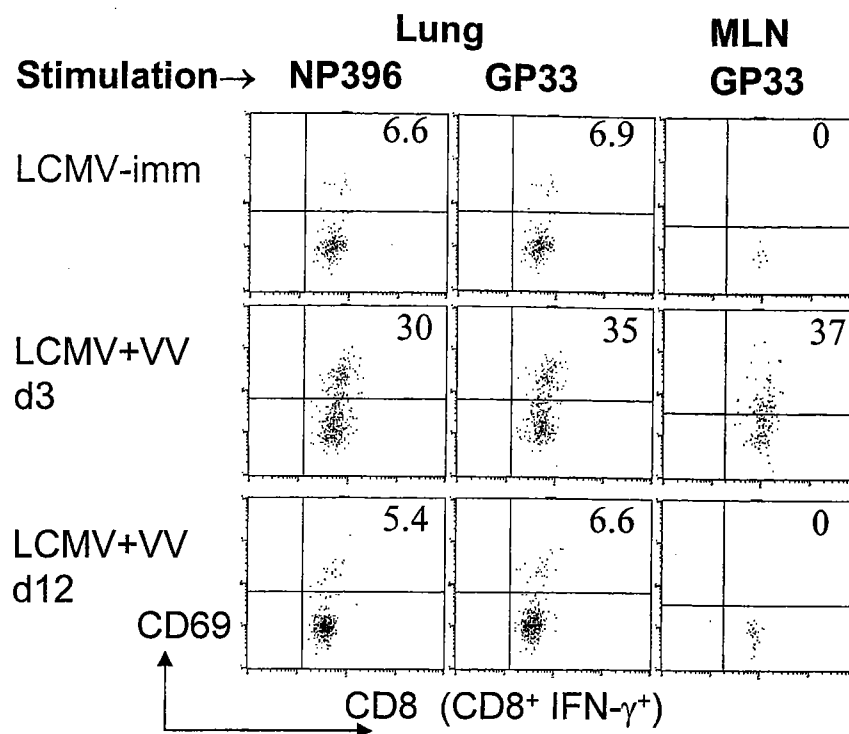


Figure 7. Increased CD69 expression on LCMV-specific memory CD8 T cells early after VV infection. Activation was assessed by staining for the early activation marker CD69. The x-axis represents CD8 staining and the y-axis represents CD69 staining of the population gated on the CD8 T cells producing IFN- γ in response to the indicated peptide. The numbers recorded in the right upper quadrants represent the percentage of CD69-staining CD8 T cells specific for the indicated epitope. There was no CD69⁺ staining of the gated cells from the control mouse lung and MLN after VV infection. These are representative data from 2 similar experiments. For each experiment lymphocytes from groups of 3 mice each were pooled prior to staining.

Table 6. Increased CD69 expression on LCMV epitope-specific memory CD8 T cells *in vivo* early after VV infection

Group	% CD69 ⁺ of CD8 ⁺ cells	% CD69 ⁺ of IgG1-MHC I dimer ⁺ CD8 ⁺ cells		
		NP396	GP33	NP205
LCMV-immune	2.3	7.1	6.7	11
LCMV-immune + VV day 3	19	43	66	29

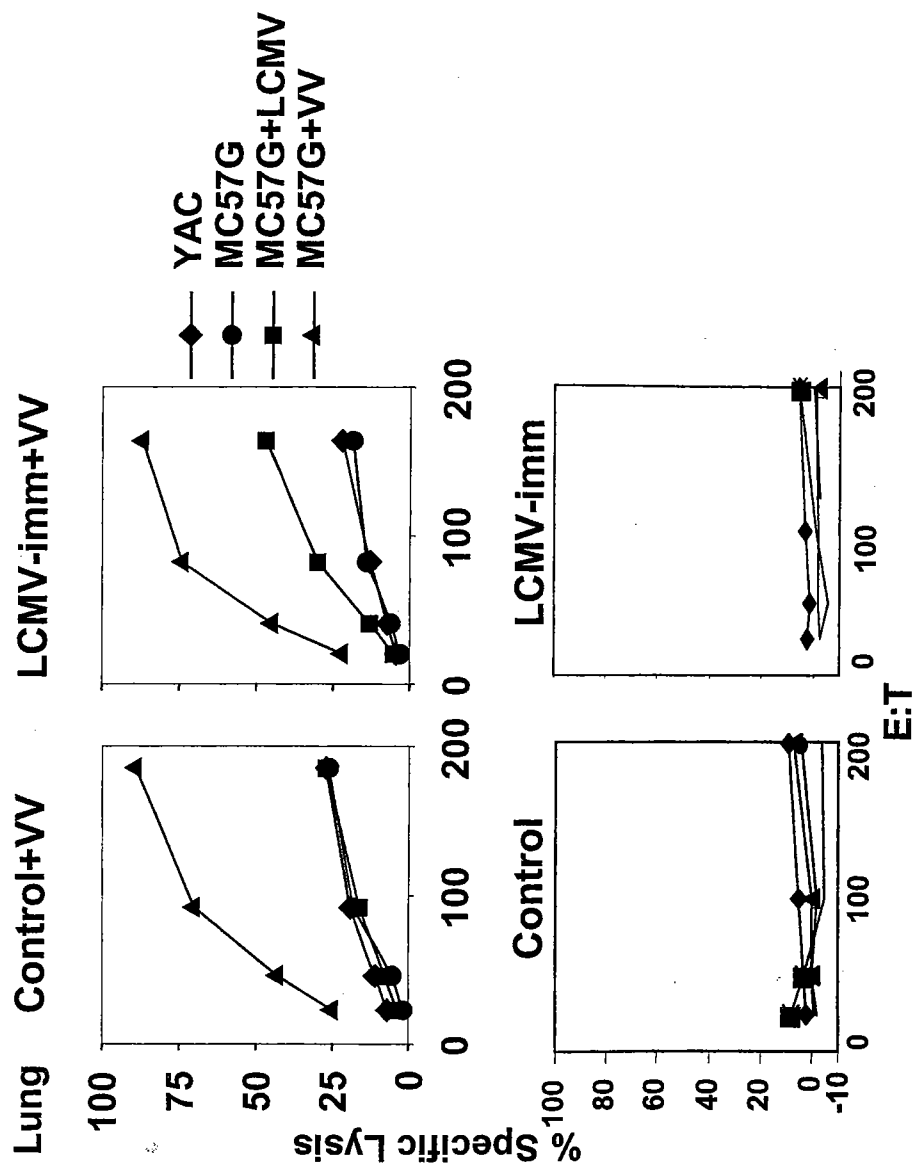
The numbers recorded represent the percentage of CD69 staining of gated CD8⁺ and LCMV epitope-specific peptide IgG1-MHC I dimer complex⁺ cells. There were no CD8⁺ and LCMV-specific dimer⁺ cells from the control mouse lung after VV infection. Lung lymphocytes from groups of 3 mice each were pooled prior to staining.

CD8 T cell populations demonstrated equivalent levels of CD69 expression (Figure 7 and not shown). It is possible that without any *in vitro* stimulation the differences in fold increases in percents of CD69+ CD8+ T cells among the three epitopes tested (Table 6) reflected the differences in the *in vivo* activation state of these three LCMV-specific memory CD8 T cell populations. Since rapid transient up-regulation of CD69 on the surface of T cells has been linked to engagement of TCR (Testi et al., 1994), up-regulation of CD69 on LCMV epitope-specific CD8 T cells in LCMV-immune mice early after VV challenge suggest that the VV-induced activation of LCMV-specific CD8 T cells may involve TCR triggering.

I. Cytolytic function in LCMV-specific memory CD8 T cells after VV challenge

I further assessed the activation status of the accumulating LCMV-specific memory CD8⁺ T cells by determining their cytolytic capacity *ex vivo* in bulk cytotoxicity assays of pooled mouse lung lymphocytes and splenocytes (Figure 8). Both LCMV-immune and control mice that had been treated with anti-NK1.1 to deplete NK cells *in vivo* mounted equivalent VV-specific CTL responses on day 6 after VV challenge. However, only in T cells from the VV-infected LCMV-immune mice did a VV-induced reactivation of LCMV-specific cytotoxicity occur (Figure 8). In nonchallenged LCMV-immune and control mice, there was no LCMV-specific lysis (Figure 8). These results further supported the findings that LCMV-specific CD8⁺ T cells were not only accumulating but were also functionally activated during acute VV infection.

Figure 8



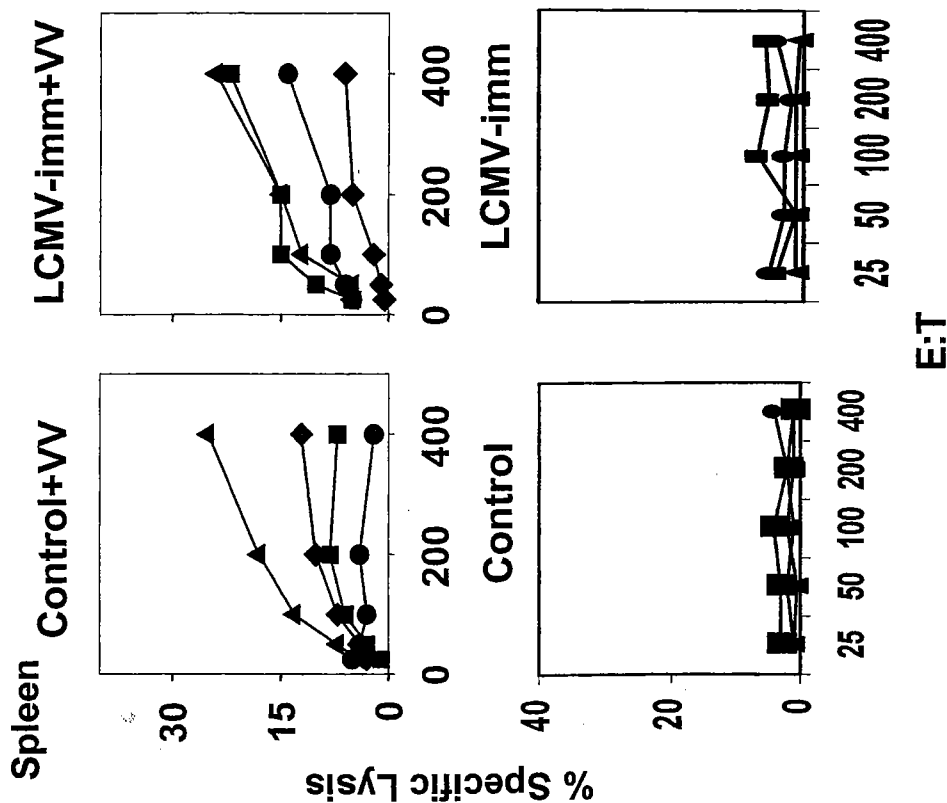


Figure 8. Activation of LCMV-specific cytolytic activity in lymphocytes isolated from the lungs and spleens of LCMV-immune mice 6 days after i.n. VV challenge. The effector cells were pooled lymphocytes isolated from 3 acute VV-infected control or LCMV-immune lungs and spleens. Both groups of mice were injected with anti-NK1.1 antibody to deplete NK cells *in vivo*. The ^{51}Cr -release CTL assay was carried out as described in Materials and Methods. These are representative data from 3 similar experiments.

J. A modified intracellular IFN- γ assay directly visualizes activation of LCMV-specific memory CD8 T cells *in vivo* by VV early in infection

Was there any direct evidence that these accumulating LCMV-specific memory CD8 T cells were functionally activated *in vivo* during VV infection? Immediately after isolation, the lung lymphocytes, without any *in vitro* stimulation or incubation, were stained for intracellular IFN- γ . Among the tested populations, NK cells (16%, 2.3×10^4 cells), CD4 T cells (0.2%, 1.0×10^3 cells), and $\gamma\delta$ T cells (2.6%, 6.0×10^2 cells) pooled from the lungs of 3 VV-infected LCMV-immune mice had produced IFN γ "*in vivo*". Day 3 is considered the peak of the host response by NK and $\gamma\delta$ T cells (Selin et al., 2001; Tay et al., 1999). However, there was no increase in IFN- γ production by these cells in LCMV-immune mice 3 days after VV challenge, when compared to the VV-infected control mice (lung lymphocytes pooled from 3 control mice: NK cells, 23%, 3.7×10^4 cells; CD4 T cells, 0.5%, 1.4×10^3 cells; and $\gamma\delta$ T cells, 1.7%, 4.0×10^2 cells; Figure 9 and Table 7). Although the percentage of IFN- γ -producing NK cells in VV-infected LCMV-immune mouse lungs was slightly lower than that in VV-infected control mouse lungs, the total number of IFN- γ -producing cells in VV-infected LCMV-immune mouse lungs was twice as high as that in VV-infected control mouse lungs (Figure 9 and Table 7). This was because of a significant increase in IFN- γ -producing CD8 T cells in VV-infected LCMV-immune mouse lungs as compared to the controls (Figure 9 and Table 7). Similar patterns were also observed in the MLN and spleen 3 days after VV challenge (Table 7). Furthermore, the CD8 T cells which were producing IFN- γ "*in vivo*" did not co-express the NK cell associated molecule, NK1.1.

In order to determine whether these IFN- γ -producing CD8 T cells were LCMV-specific, the lung lymphocytes immediately after isolation and without any *in vitro*

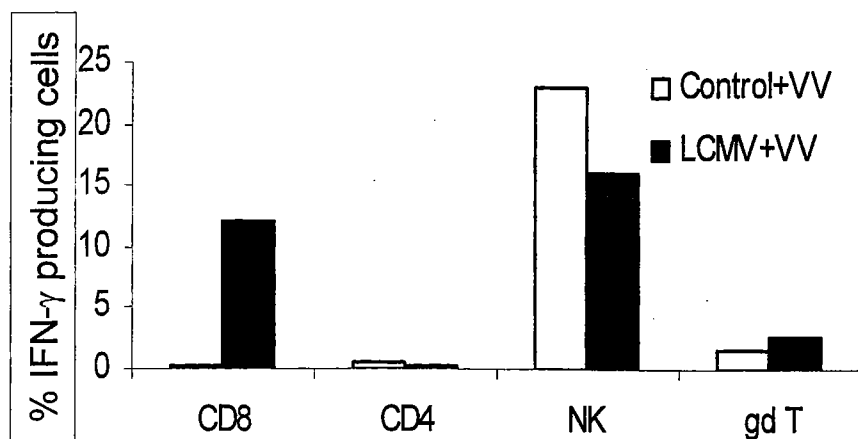


Figure 9. *in vivo* IFN- γ -producing cells in the LCMV-immune mouse lungs 3 days after VV challenge.

Lymphocytes isolated from mouse lungs were immediately placed at 4 °C and stained with anti-CD3, CD8, CD4, NK1.1, and $\gamma\delta$ TCR mAbs, as well as anti-IFN- γ mAb without any stimulation *in vitro*. Y-axis indicates the percentage of cells stained with intracellular IFN- γ . These are representative data from one of 2 similar experiments. For each experiment lymphocytes from groups of 3 mice were pooled prior to staining.

Table 7. Production of IFN- γ *in vivo* in LCMV-immune mice 3 days after VV challenge¹

Organ	Exp #	Groups	CD8 ⁺ IFN- γ ⁺		CD4 ⁺ IFN- γ ⁺		NK1.1 ⁺ IFN- γ ⁺		$\gamma\delta$ ⁺ IFN- γ ⁺		Total IFN- γ ⁺	
			%	# ²	%	#	%	#	%	#	%	#
Lung	1	LCMV-imm	8.4	2.7 $\times 10^4$	0.7	1.0 $\times 10^3$	9.3	1.4 $\times 10^4$				4.2 $\times 10^4$
		Control	1.4	1.5 $\times 10^3$	0.9	1.4 $\times 10^3$	10.6	1.8 $\times 10^4$				2.1 $\times 10^4$
	2	LCMV-imm	12	4.3 $\times 10^4$	0.2	1.0 $\times 10^3$	16.2	2.3 $\times 10^4$	2.6	6.0 $\times 10^2$		6.8 $\times 10^4$
		Control	0.3	6.0 $\times 10^2$	0.5	1.4 $\times 10^3$	23.8	3.7 $\times 10^4$	1.7	4.0 $\times 10^2$		3.9 $\times 10^4$
MLN	1	LCMV-imm	0.3	2.7 $\times 10^3$	0.1	6.5 $\times 10^2$	1.9	1.3 $\times 10^2$				3.5 $\times 10^3$
		Control	0	0	0.1	6.0 $\times 10^2$	4.1	2.5 $\times 10^2$				8.5 $\times 10^2$
	2	LCMV-imm	0.1	1.9 $\times 10^2$	0	0	0.8	1.2 $\times 10^2$	0	0		3.1 $\times 10^2$
		Control	0	0	0.04	1.4 $\times 10^2$	1.7	2.2 $\times 10^2$	0	0		3.6 $\times 10^2$
Spleen	1	LCMV-imm	0.4	2.5 $\times 10^4$	0	0	1.4	1.2 $\times 10^4$				3.7 $\times 10^4$
		Control	0.1	6.5 $\times 10^3$	0	0	1.1	1.2 $\times 10^4$				1.8 $\times 10^4$
	2	LCMV-imm	1.0	5.0 $\times 10^4$	0.1	8.3 $\times 10^3$	3.6	9.0 $\times 10^3$	0.2	8.7 $\times 10^2$		6.8 $\times 10^4$
		Control	0.1	2.5 $\times 10^3$	0.1	6.3 $\times 10^3$	7.9	2.5 $\times 10^4$	0.3	1.1 $\times 10^3$		3.5 $\times 10^4$

1, with anti-CD3 Ab staining; 2, absolute cell number.

stimulation or incubation were stained for intracellular IFN- γ and with LCMV epitope-specific MHC dimers on ice (Figure 10). There was no *in vivo* IFN- γ production by LCMV-epitope specific memory CD8 T cells isolated from resting LCMV-immune mice. As compared to minimal "*in vivo*" IFN- γ production by 0.7% of CD8 T cells (4.2×10^2 cells) from the VV-infected control mouse lungs, 16% of CD8 T cells (6.2×10^4 cells) from the LCMV-immune mouse lungs were producing IFN- γ "*in vivo*" 3 days after VV challenge. Among them, 29% of the NP205-specific, 25% of the GP33-specific and 16% of the NP396-specific CD8 memory T cells in the lung were producing IFN- γ "*in vivo*" (Figure 10).

This experiment with MHC dimers charged with three of the six LCMV-specific epitopes accounted for 26% of the IFN- γ -producing cells being LCMV-specific (Table 8 Exp. 3). Two additional similar experiments with MHC dimers to define four of the LCMV epitope-specific responses (NP205, NP396, GP33 and GP34) showed that 11% and 10% of the lung CD8⁺ T cells produced intracellular IFN- γ 3 days after VV infection, and that 58% and 42% of those responses, respectively, could be attributed to CD8⁺ T cells specific to those LCMV epitopes (Table 8 Exp 5 and 6). These experiments may indicate that, at this time after VV infection, most of the IFN- γ -producing cells were LCMV-specific, because not all the LCMV epitopes are tested for IFN- γ production. Two of the immunodominant epitopes were not tested, and it is unlikely that low-affinity responses or responses by receptor down-modulated T cells would be recorded. This, of course, does not rule out the possibility that some of this response was mediated by memory T cells not specific to LCMV or newly activated naïve T cells specific to VV.

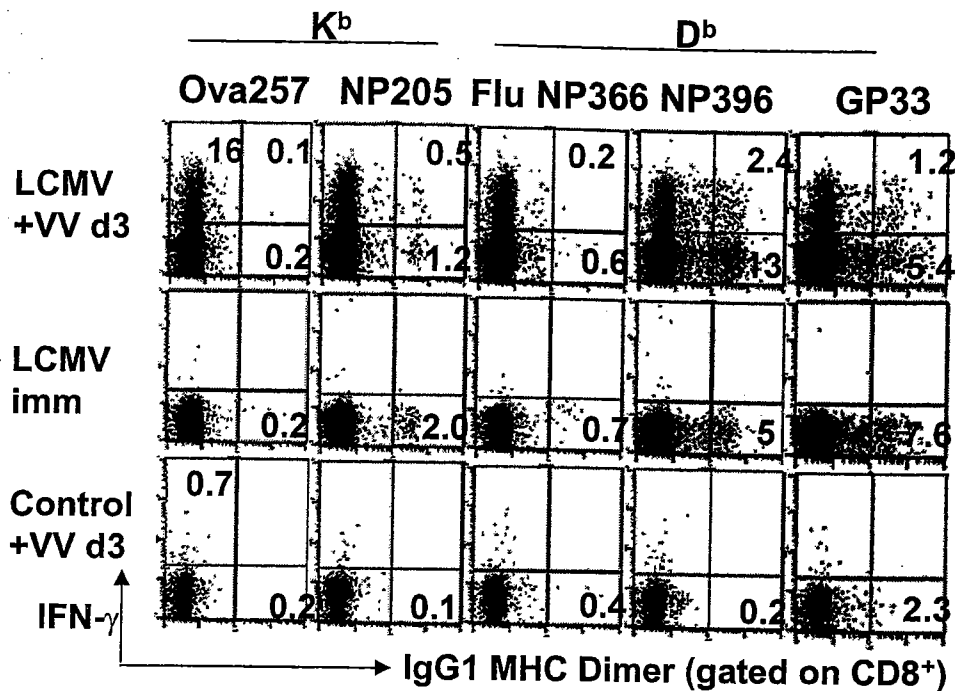


Figure 10. Increased "*in vivo*" IFN- γ production by LCMV-specific CD8 memory T cells early after VV infection. Mice were infected as indicated. Lymphocytes isolated from mouse lungs were immediately placed at 4 °C and stained with anti-CD8 mAb, 3 of 6 potential LCMV epitope-specific MHC dimers (NP205, NP396, and GP33) for which we had functioning dimers and anti-IFN- γ mAb without any stimulation *in vitro*. The data presented in this figure have been gated on CD8⁺ T cells. The numbers in each quadrant indicate the percentage of CD8 T cells stained with the indicated MHC dimer and/or intracellular IFN- γ . These are representative data from one of 3 similar experiments. For each experiment lymphocytes from groups of 3 mice each were pooled prior to staining.

Table 8. *In vivo* IFN- γ production by LCMV epitope-specific memory CD8 T cells in the lung after VV challenge

Exp #	Group	CD8 ⁺ IFN- γ ⁺ %	% Dimer ⁺ IFN- γ ⁺ / % Dimer ⁺ (proportion of <i>in vivo</i> IFN- γ -producing epitope-specific CD8 T cells)						Total ³
			K ^b			D ^b			
			Ova257	NP205	GP34	Flu NP366	NP396	GP33	
1 ¹	LCMV-imm +VV d3	9.6					1.6/8.1 (19%)		17 (1)
	LCMV-imm 0	0					0/7.4		
2 ²	LCMV-imm +VV d3	16		0.3/1.2 (25%)			1.8/5.8 (31%)	0.7/3.0 (23%)	18 (3)
	LCMV-imm 0	0		0/0.8			0/7.4	0/2.8	
3	LCMV-imm +VV d3	16	0.1/0.3	0.5/1.7 (29%)		0.2/0.8	2.4/15.4 (16%)	1.2/6.6 (25%)	26 (3)
	LCMV-imm 0	0	0/0.1	0/1.7		0/0.2	0/7.4	0/2.8	
4	LCMV-imm +VV d3	6.7	0/0.3	0.2/1.5 (13%)		0/0	1.2/5.6 (21%)	0.4/4.3 (9.3%)	27 (3)
	LCMV-imm 0	0	0/0.3	0/1.5		0/0	0/4.4	0/2.2	
5	LCMV-imm +VV d3	11	0/0.1	0.3/3.7 (8.8%)	1.9/14.6 (13%)	0/0.2	3.2/12 (27%)	1.1/4.9 (22%)	58 (4)
	LCMV-imm 10	10	0/0.05	0.8/2.4 (33%)	0.6/9.9 (6%)	0/0.3	1.7/18.5 (9.2%)	1.1/7.5 (15%)	
6	LCMV-imm +VV d3								42 (4)

1, tetramer; 2, dimer (Experiment 2 to 6); 3, proportion of IFN- γ -producing LCMV-specific CD8 T cells out of the total IFN- γ -producing CD8 T cells. The numbers in the parentheses were the numbers of the tested LCMV-specific epitopes.

The control H2K^b-dimer loaded with ovalbumin-specific peptide (OVA₂₅₇₋₂₆₄) bound < 0.2% of CD8 T cells. The control D^b-dimer loaded with the influenza A virus peptide (NP₃₆₆₋₃₇₄) bound < 0.8% of CD8 T cells, and they produced minimal IFN- γ . Some degree of recognition of influenza NP366-pulsed targets by LCMV-specific CTLs has been observed (Selin and Welsh, 1997). A low level (2.3%, Figure 10) binding of the GP33-specific dimers to CD8 T cells from the lungs of VV-infected control mice also occurred with CD8 T cells from the lungs of non-infected control mice, suggesting that GP33-specific dimers might have a low level of non-specific binding, or that GP33 might cross-react with VV and some self antigens.

K. Enhanced IFN- γ production in LCMV-immune mice early after VV challenge

RNase protection assays showed 4.7-fold higher levels of IFN- γ mRNA in the LCMV-immune mouse lungs, compared to VV-challenged control mouse lungs, 3 days after VV challenge (relative intensity of IFN- γ : LCMV+VV, 0.33 ± 0.01 ; control +VV, 0.07 ± 0.003 , $P=0.00003$) (Figure 11a). In the unchallenged LCMV-immune mouse lungs, compared to the nonchallenged control mouse lungs, a low basal amount of IFN- γ mRNA was observed. In addition, three- to five-fold higher concentrations of IFN- γ protein—as detected by enzyme linked immunosorbant assay (ELISA)—were observed in the sera of VV-challenged LCMV-immune mice (Figure 11b). The difference in IFN- γ levels between VV-infected LCMV-immune and control groups was seen as early as 3 days after VV infection. The greatest difference occurred by day 5 after VV infection. In contrast, after VV challenge two-fold higher mRNA levels of IL-6, a potent

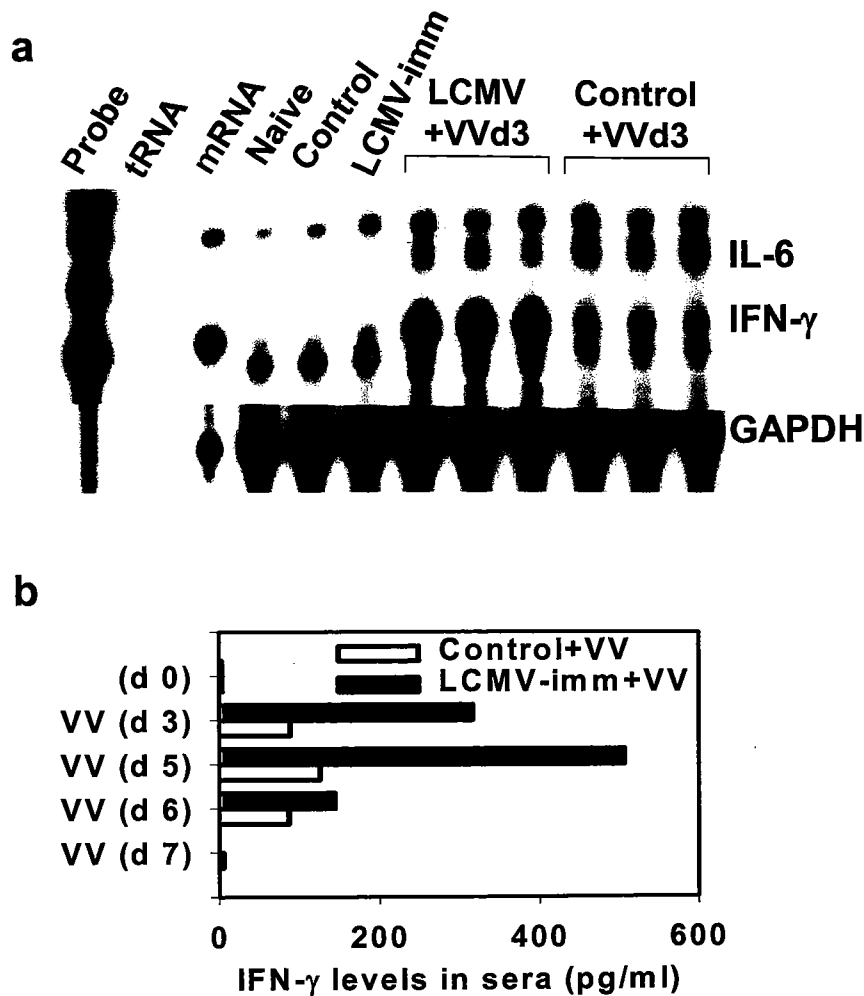


Figure 11. Increased IFN- γ in LCMV-immune mice after VV challenge as assessed by (a) an RNase protection assay of cytokine mRNA levels in the lung tissue and (b) an ELISA assay that measured IFN- γ in pooled sera. tRNA, negative control; mRNA, positive control; GAPDH, the housekeeping gene (used to normalize mRNA levels).

pro-inflammatory cytokine, were observed in control mouse lungs compared to the LCMV-immune mouse lungs (relative intensity of IL-6: LCMV+VV, 0.04 ± 0.0003 ; control +VV, 0.08 ± 0.01 , $P < 0.05$) (Figure 11a). Higher amounts of serum IL-6 were also found in the VV-infected control mice (42 pg/ml, sera pooled from three mice) compared to the VV-challenged LCMV-immune mice (12 pg/ml, sera pooled from three mice). Because IL-6 is a pro-inflammatory cytokine (Ramshaw et al., 1997), these results suggested that a stronger acute inflammatory response took place in the control mice and that previous immunity to LCMV markedly altered the cytokine responses induced by VV infection.

L. An important role for IFN- γ in mediating protective immunity to VV in LCMV-immune mice

IFN- γ has marked impact on VV replication (Muller et al., 1994). To further test whether the increased IFN- γ produced in the LCMV-immune mice was important in this protective immunity, LCMV-immune mice were treated with anti-IFN- γ and infected with VV. In two similar experiments (Table 9), anti-IFN- γ treatment, in comparison to control mAb, resulted in significant 4- and 3.2-fold increases in VV titers in the lungs, and 5- and 15-fold increases in VV titers in the pooled MLNs of LCMV-immune mice. The importance of IFN- γ in regulating VV synthesis in LCMV-immune mice has also been documented in mice during i.p. VV infections. 129/SEV mice immune to LCMV developed protective heterologous immunity to VV, similar to that in the C57BL/6 mice, whereas IFN- γ R KO 129/SEV mice immune to LCMV did not resist VV infection (Selin et al., 1998).

Table 9. Enhanced titers of VV in the LCMV-immune mice on treatment with α IFN- γ mAb

Exp #	Organ	Treatment (n = number of mice per group)	Virus Titer (mean log ₁₀ PFU/organ \pm SEM) ^b	P value (α IFN- γ v.s. IgG1 control)
No. 1	Lung	α IFN- γ (n = 6)	6.8 \pm 0.1	P = 0.002
		IgG1 control (n = 6)	6.2 \pm 0.1	
	Spleen	α IFN- γ (n = 6)	1.7 \pm 0.3	P < 0.05
		IgG1 control (n = 6)	<1	
	MLN (pooled)	α IFN- γ (n = 6)	1.7	
		IgG1 control (n = 6)	1	
No. 2	Lung	α IFN- γ (n = 5)	6.8 \pm 0.1	P = 0.02
		IgG1 control (n = 5)	6.3 \pm 0.1	
	MLN (pooled)	α IFN- γ (n = 5)	2.7	
		IgG1 control (n = 5)	1.5	

In vivo depletion of IFN- γ was performed by i.v. (day 0 and 3) and i.p. (daily) injection of 100 μ l of an ascites of rat anti-IFN- γ mAb (R4-6A2) or a control rat IgG1 isotype (clone A110-1, Pharmingen) following VV challenge. At day 6.5 after VV infection, the lungs, MLNs and spleens were harvested and underwent virus titration and histological analysis.

M. Altered immunopathology in VV-infected LCMV-immune mouse lungs

To examine whether immunity to LCMV would influence VV-induced pathology in the lung, tissue sections were stained with hematoxylin and eosin (Figure 12). Compared to sections from naïve mouse lungs (Figure 12a), sections from LCMV-immune mouse lungs (Figure 12b) showed some mononuclear (MN) infiltrating cells and occasional lymphocyte aggregates in the interstitium, a pattern that can be seen in older naïve mice in any clean mouse colony. The majority of the LCMV-immune lung architecture was normal. Seven days after VV infection, the lungs from the LCMV-immune and control mice had completely different pathologies (Figure 12c,e and Table 10). We found that 72% of the control mouse lungs had severe alveolar edema (Figure 12c and Table 10): an accumulation of the extravascular fluid in the air spaces, which presumably disturbed gas exchange. In addition, the VV-infected control mouse lungs mainly showed acute mixed inflammatory infiltrates (AMI) with polymorphonuclear (PMN) and MN cells in the peribronchial areas (86%) and interstitium (71%), as well as in the perivascular areas (71%) (Figure 12d and Table 10). In contrast, the lungs of LCMV-immune mice challenged with VV showed a prominent lymphocytic response, which may have aided in the clearance of VV. In addition, 80% of the LCMV-immune mouse lungs had marked induction of the lung lymphoid system (Figure 12e and Table 10), as shown by prominent bronchus-associated lymphoid tissue (BALT). BALT was defined by nodules of lymphoid tissue in the bronchial lamina propria near the branch points of an airway or between the bronchus and an artery (Pabst, 1992). Although acute LCMV infection induced a minimal transient BALT, there was no BALT present in the resting LCMV-immune mouse lungs (Figure 12b). Prominent chronic MN infiltration

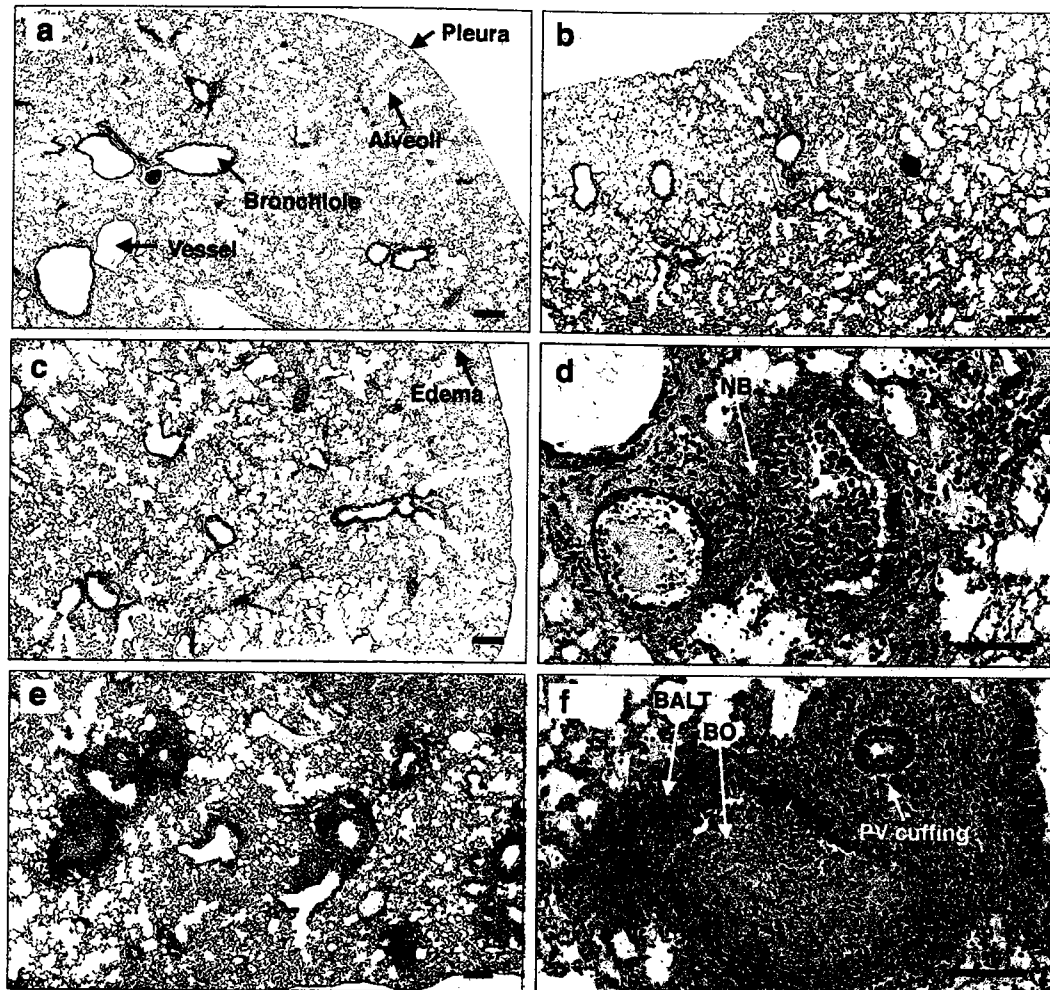


Figure 12. Enhanced lymphocytic infiltration in the lungs of LCMV-immune mice after infection with VV. Lung sections from mice that did not receive VV (a-b) or from VV-infected mice 7 days PI (c-f) were stained with H+E. a) Naive mouse lungs had normal architecture. b) LCMV-immune mouse lungs had very mild lymphocytic infiltrates in the interstitium. c) VV-infected control mouse lungs mainly showed severe alveolar edema (pink material in air spaces), and d) acute necrotizing bronchiolitis (NB) and peribronchiolitis as well as neutrophilic infiltrates in the interstitium. e) VV-infected LCMV-immune mouse lungs had significantly increased BALT around the airways and no alveolar edema; f) BALT surrounded an airway with bronchiolitis obliterans (BO), and also present was perivascular (PV) lymphocytic cuffing. Bars = 100 μ m.

Table 10. Altered immunopathology in the i.n. VV-infected LCMV-immune mouse lungs

Lung Compartments	Pathological Features	% Mice with lung involvement§							
		Control+VV (n=7) [#]				LCMV-imm + VV (n=5) [#]			
		0	+	++	+++	0	+	++	+++
Alveoli	Edema	14	14	29	43	40	40	20	
BALT	Extent of BALT	86	14				20	40	40
Airways	Necrotizing bronchiolitis	29	43	14	14		20	80	
	Peribronchial AMI†	14	58	14	14	100			
	Bronchiolitis obliterans	100				60	20	20	
Interstitialium	CMI‡	100				20	20	40	20
	AMI	29	29	13	29	60	40		
Pleura	CMI	100					100		
Vasculature	Lymphocytic cuffing	100				40	60		
	Necrotizing vasculitis	86		14		40	60		
	Edema + AMI	29	57		14	100			

Seven days after VV infection the mouse lungs were harvested, sectioned and stained (H+E), as described in Materials and Methods. These data are representative of 5 similar experiments.

†, AMI, acute mixed infiltrate (polymorphonuclear and mononuclear cells);

‡, CMI, chronic mononuclear infiltrate (lymphocytes and macrophages);

§, Grading of the pathological changes in the lung: based on the distribution and the severity of disease from 0 to +++ (0, within normal limit; +, 1-9%, ++, 10-49%, and +++, ≥50% involvement of the lung parenchyma).

#, the number of mice in each group.

(CMI) with lymphocytes and macrophages was present in the interstitium and pleura of almost all the VV-infected LCMV-immune mouse lungs.

In addition, 60% of the LCMV-immune lungs had perivascular lymphocytic cuffing, as defined by lymphocyte accumulation around the vessels (Figure 12f and Table 10). LCMV-immune mouse lungs challenged with VV also showed the less common pathology of necrotizing vasculitis (60%) and bronchiolitis obliterans (40%) (Figure 12f), which, in humans, are thought to be immune-mediated diseases of unknown etiology. By 7 days after VV infection, lungs from both groups showed necrotizing bronchiolitis, one of the main lesions of the disease (Figure 12d,f), as defined by the presence of necrotic bronchiolar epithelium with PMNs and debris. Thus, altered immunopathological features were seen in the VV-infected LCMV-immune mice lungs, which showed a prominent lymphocytic response, whereas the VV-infected control mouse lungs mainly had acute neutrophilic infiltrates and severe edema.

N. Bronchiolitis obliterans and BALT persist in the LCMV-immune mouse lungs long after VV challenge

To further study the effect of prior immunity to LCMV on the altered lung immunopathology upon VV infections, I examined lung histopathology in LCMV-immune mice at later time points after the VV challenge. As shown above, 7 days after VV challenge, some of the VV-challenged LCMV-immune mouse lungs developed bronchiolitis obliterans (Figure 12f and Table 10). By day 14 and 30 post VV postinfection, moderate bronchiolitis obliterans was still present in 4 out of 5 VV-challenged LCMV-immune lungs (Table 11a).

Table 11a. Altered immunopathology persists in the LCMV-immune lungs long after VV challenge

Lung Compartments	Pathological Features	Severity grading ^c		
		Day 14	Day 14	Day 30
		Control + VV (n = 2) ^d	LCMV-immune + VV (n = 2)	Control + VV (n = 3)
Alveoli	Edema	3	3	LCMV-immune + VV (n = 3)
BALT	Extent of BALT	3	2	0.3 (0 - 1)
Airways	Bronchiolitis	1.0 (0 - 2)	0.3 (0 - 1)	0.3 (0 - 1)
	Peribronchial AMI ^a	1.0 (0 - 2)	1.0 (0 - 2)	2.3 (2 - 3)
	Bronchiolitis obliterans	0.5 (0 - 1)	1	0.3 (0 - 1)
				0
Interstitial	CMI ^b	1	1.5 (1 - 2)	0.7 (0 - 1)
	AMI		0.5 (0 - 1)	1
Pleura	CMI	0.5 (0 - 1)	1	1.3 (1 - 2)
Vasculature	Lymphocytic cuffing and tufting	3	2	0.6 (0 - 1)
	Necrotizing vasculitis		0.5 (0 - 1)	1
	Edema			0.3 (0 - 1)

Table 11b. Presence of BALT in the lungs of LCMV- or VV-immune mice after homologous virus challenge

Lung Compartments	Pathological Features	Severity grading		
		LCMV-immune + LCMV	VV-immune + VV	
		Day 7 (n = 2)	Day 30 (n = 2)	Day 7 (n = 1) Day 69 (n = 3)
BALT	Extent of BALT	2.0 (1 - 3)	0.5 (0 - 1)	2 1.6 (1 - 2)

After viral infection, the mouse lungs were harvested, sectioned and stained (H+E), as described in Materials and Methods. a, AMI, acute mixed infiltrate (polymorphonuclear and mononuclear cells); b, CMI, chronic mononuclear infiltrate (lymphocytes and macrophages); c, Grading of the pathological changes in the lung: based on the distribution and the severity of disease from 0 to 3 (*blank* or 0, within normal limit; 1, 1-9%, 2, 10-49%, and 3, $\geq 50\%$ involvement of the lung parenchyma). d, the number of mice in each group. e, Data are presented as average of scores from multiple mice per treatment, and the number in parentheses after each average score represents the range of scores from mice challenged with the virus indicated.

Another interesting finding was persistence of prominent BALT in the lungs of VV-challenged LCMV-immune mice. Expansion of BALT occurred in the VV-infected LCMV-immune lungs as early as day 5 postinfection (see Chapter V Table 20). The extensive BALT tended to persist, as there was still a substantial BALT in VV-challenged LCMV-immune lungs 30 days after VV infection (Table 11a) and even 43 days after VV infection (not shown). As shown in Table 11a, enhanced BALT developed but only late (greater than 14 days) after VV infection in the control mouse lungs. Thirty days after VV infection, only very mild BALT presented in the VV-challenged control mouse lungs (Table 11a).

To define whether the persistent BALT is a unique feature in the LCMV-immune mouse lungs with heterologous VV infection, I also examined histology in LCMV-, and VV-immune mouse lungs after homologous secondary viral challenge. The secondary LCMV infection induced a transient mild to moderate BALT, and 30 days after the secondary LCMV infection, only very mild BALT persisted in the LCMV-challenged LCMV-immune mouse lungs (Table 11b). The secondary VV infection induced moderate BALT as early as 7 days after challenge, and BALT persisted in the lung even 69 days after secondary challenge (Table 11b). Therefore, expansion and persistence of BALT observed in the lungs of VV-challenged LCMV-immune mice were more like the changes occurring in the lungs of VV-challenged VV-immune mice. This suggests that VV may be a good inducer of BALT with a high frequency of memory T cells in the immune mouse lungs and there may be a distinct mechanism to control the size of BALT, which is an inducible lymphoid tissue. In one experiment with day 14-VV-infected LCMV-immune mice, I found that CD8+ T cell clusters (Figure 13b) and scattered CD4 T cells (Figure

Figure 13

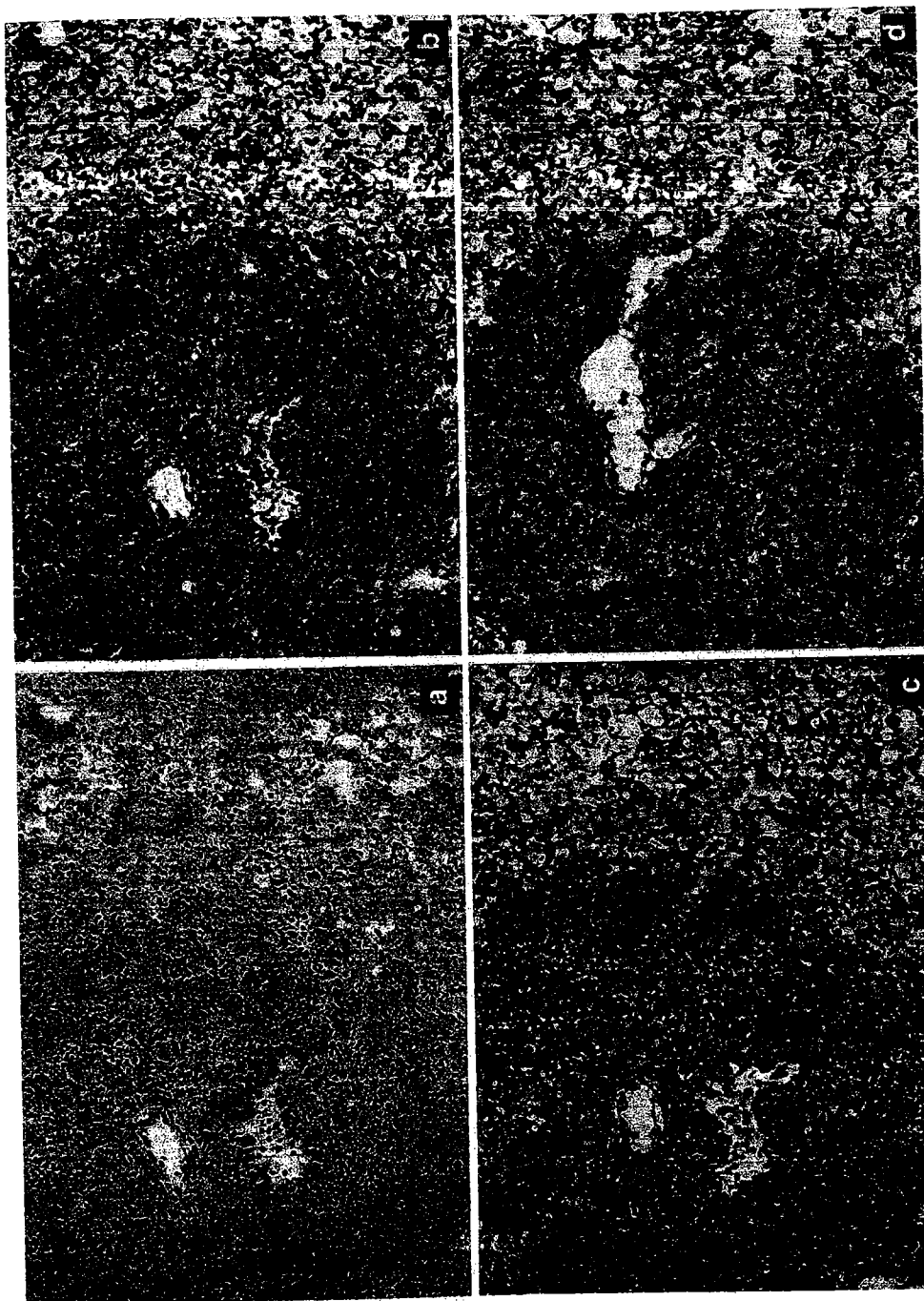


Figure 13. The predominant infiltrates in the BALT of the LCMV-immune mouse lung challenged with VV are T cells.

Fourteen days after VV challenge, lungs from LCMV-immune mice were harvested, and frozen sections (5 mm) were cut and fixed, then undergone immunohistochemistry staining. **(a)** shows hematoxylin and eosin staining. The serial sections, which show similar pulmonary architecture, were incubated with biotin-labeled mAbs to CD3 (clone 145-2C11), **(b)** CD8 (clone 53-5.8), **(c)** CD19 (Clone 1D3), or **(d)** CD4 (clone RM4-5). Sections were then stained with streptavidin-HRP and DAB solution. The sections were counterstained with. Magnification: 50× of the objective of the digital light-microscope.

13d) surrounded a bronchiolar and vascular bundle, while no CD19+ B cells were found in the BALT (Figure 13c). These results suggested that the predominant infiltrates in the BALT of the VV-challenged LCMV-immune mouse lung were T cells.

O. *In vivo* depletion of IFN- γ results in severe pulmonary edema and no induction of BALT in VV-infected LCMV-immune mice

Because the LCMV-specific memory CD8⁺ T cells produced IFN- γ in the lung of VV-challenged mice, we questioned whether IFN- γ played a role in the altered lung pathology. Compared to the VV-infected LCMV-immune mice treated with control IgG1 (Figure 14a), *in vivo* depletion of IFN- γ in the VV-infected LCMV-immune mice by intravenous (i.v.) and i.p. injection of anti-IFN- γ resulted in severe edema and no induction of BALT (Figure 14b). This pathology was similar to the changes observed in the VV-infected control mouse lungs (Figure 12c), which suggested that IFN- γ might play a role in recruiting the lymphocytes to the lungs and thus alter the pathology in the VV-infected LCMV-immune mouse lungs. This is consistent with studies showing that IFN- γ participates in inducing chemokines to recruit activated lymphocytes to the liver, uterus and ovary, the sites of VV replication (Mahalingam et al., 2000). Therefore, the enhanced IFN- γ response in immune mice may play a role in the induction of immunopathology.

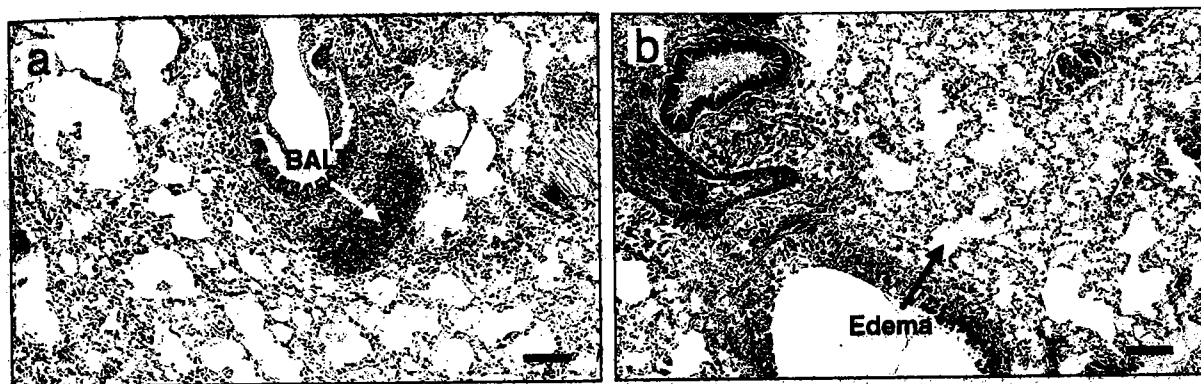


Figure 14. Severe edema and impaired lymphocytic infiltration in the lungs of VV-infected LCMV-immune mice upon treatment with anti-IFN- γ mAb. Lung sections from mice that had been infected with VV 7 days previously were stained with hematoxylin and eosin. (a) VV-infected LCMV-immune mice were treated with control IgG1. (b) *In vivo* depletion of IFN- γ in VV-infected LCMV-immune mice by daily i.v. and i.p. injection of anti-IFN- γ for 6 days resulted in severe pulmonary edema and no induction of BALT. Bars=100 μ m.

P. Detecting *in situ* LCMV-specific CD8 T cells in BALT of VV-infected LCMV-immune mouse lung by using MHC tetramer and confocal laser scanner microscopy

In order to determine if the BALT in the lungs of LCMV-immune mice acutely infected with VV was composed of LCMV-specific memory CD8 T cells, I stained lung sections *in situ* with LCMV epitope-specific MHC tetramers. I chose NP396-specific tetramers, which were easily made because NP396-404 peptide binds to H2D^b at 1000-fold higher affinity than other LCMV epitope peptides (Gairin et al., 1995). Because BALT is not prominent in VV-infected control mouse lungs, there were few clusters of CD8 T cells in those lungs (Figure 15b) as compared to the LCMV-immune lungs, which had prominent BALT (Figure 15a). Those few CD8 T cells present in the VV-infected control mouse lungs did not co-stain with NP396 tetramer (Figure 15b). In contrast, in the LCMV-immune lungs after VV challenge there were many CD8 T cells which co-stained with NP396 tetramer (Figure 15a), demonstrating that LCMV-specific CD8 T cells were recruited to and contributed to the formation of the prominent BALT, one of the hallmarks of the altered pathology present in these mice. A similar staining pattern was also found in the VV-infected LCMV-immune mouse lung frozen sections by using NP396-specific IgG-MHC I dimers (not shown).

Q. Brief summary

I have shown in this chapter that mice with respiratory infection with LCMV can clear the virus in the lung and enter a resting immune healthy state. When these LCMV-immune mice are challenged intranasally with VV, prior immunity to LCMV provides

Figure 15

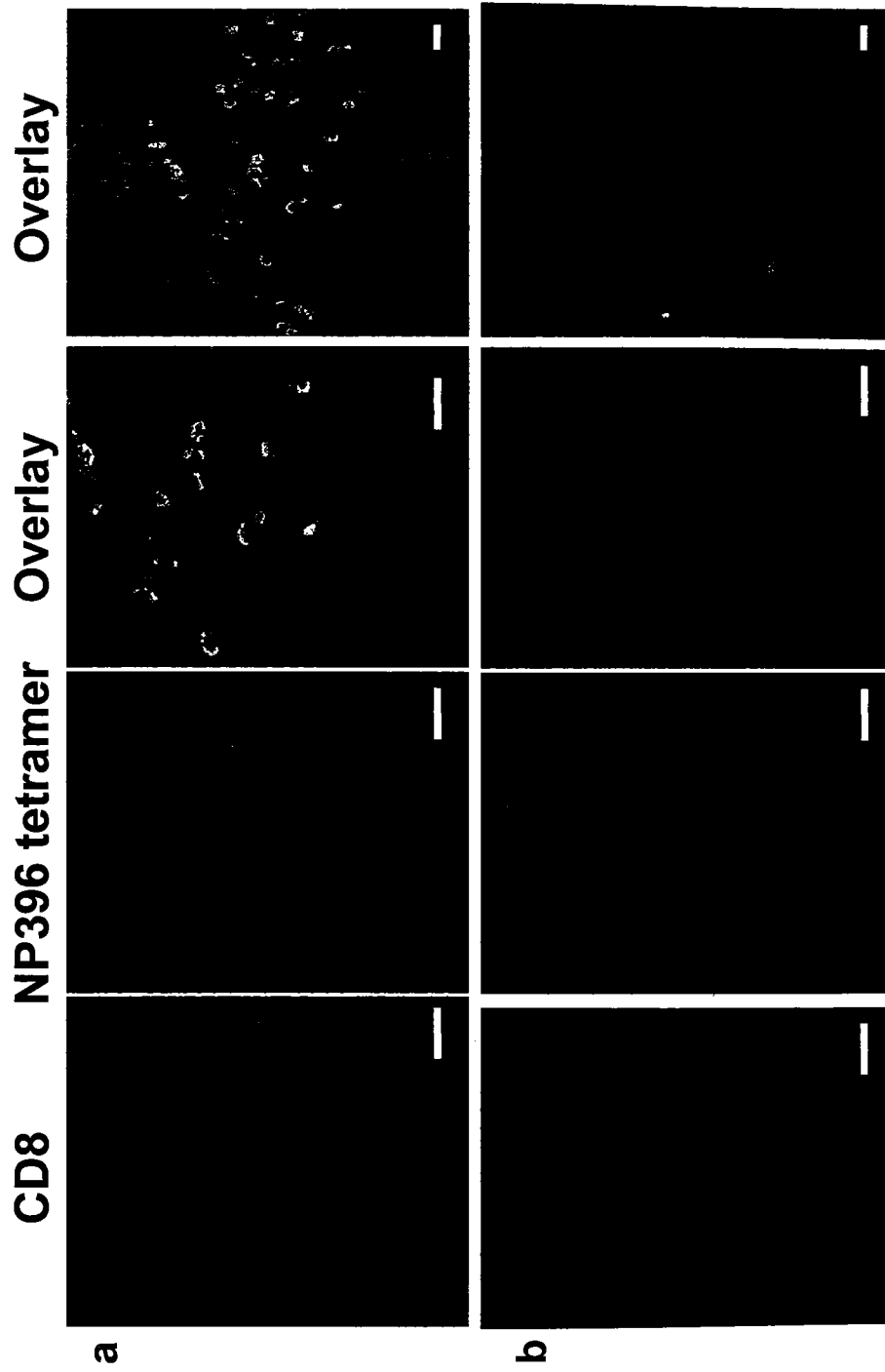


Figure 15. *In situ* detection of LCMV epitope-specific CD8 T cells in lung tissue with NP396-specific tetramer. Seven days after VV challenge, lungs from the (a) LCMV-immune and (b) control mice were harvested; and frozen sections (20 μ m) were cut, stained and displayed as single color with anti-CD8 mAb, NP-396 MHC tetramer, and colored overlay (yellow) of CD8 staining (green) and NP-396 MHC tetramer staining (red). The last panels of a and b show a larger area. These data are representative of 3 similar experiments. Bars = 25 μ m.

protection against a lethal VV challenge, and the decrease in mortality in the VV-infected LCMV-immune mice is associated with a decreased VV load throughout the course of the VV infection. Moreover, I provide data that this protective heterologous immunity against VV is dependent on LCMV-immune leukocytes.

In this chapter, I systematically examined what impact heterologous virus challenge had on memory CD8⁺ T cell populations. This impact resulted in: (i) the up-regulation of CD69 expression, the activation of cytotoxic function and IFN- γ production of LCMV-specific CD8 memory T cells upon VV challenge; (ii) the selective expansion and modulation of the original LCMV-specific memory CD8 T cell repertoire; and (iii) the distinct organ-dependent redistribution of these defined antigen-specific heterologous memory T cell populations. These responses were associated with IFN- γ -dependent enhanced protective immunity and greatly altered immunopathology. Taken together, prior immunity to LCMV has a profound effect on the disease induced by VV. This heterologous immunity can be another important factor influencing the severity and ultimate outcome of viral infections.

CHAPTER IV

THE MECHANISMS INVOLVED IN ACTIVATION AND MODULATION OF MEMORY CD8 T CELLS DURING THE HETEROLOGOUS VIRAL INFECTIONS

The next logical question is what are the mechanisms involved in activation and modulation of memory CD8 T cells during the acute heterologous viral challenge. One possible mechanism is cross-reactive stimulation through TCR engagement. A TCR that recognizes a given MHC-presented peptide might also recognize other peptides that fit the appropriate MHC motif and have, projecting from the antigen-binding groove, amino acid side chains that are able to stimulate the TCR. Another possible mechanism for activation and modulation of memory CD8 T cells is Ag-independent bystander activation by cytokines. During viral infection, there is an induction of type I IFN, which can then induce IL-15. Memory CD8 T cells can be induced into cell cycle by IL-15 (Tough et al., 1999). IL-12, another important cytokine controlling many viral and mycobacterial infections, has recently been reported to drive memory CD8 T cells into cell division *in vivo*, and this effect is IFN- γ -dependent (Tough et al., 2001). A third potential mechanism involved in modulation of memory CD8 T cells during the heterologous viral infection is the migration of memory T cells, which express distinct chemokine receptors (Mahalingam et al., 2000), into areas of inflammation in a non-specific manner. Studies have shown that putatively non-cross-reactive OVA-specific memory-phenotype transgenic T cells migrate into the lung early during influenza A virus infection and thereafter disappear instead of expanding in number (Topham et al., 2001).

In this chapter, I will show that upon heterologous VV challenge there is preferential expansion in number of LCMV-specific memory CD8 T cells with discrete specificities. This selectivity suggests that cross-reactive responses played a role in this expansion. VV peptides partially homologous to LCMV NP205 stimulate LCMV NP205-specific CD8 T cells, suggesting that NP205 may be a cross-reactive epitope. Results from poly I:C treatment of LCMV-immune mice and VV challenge of IL-12KO mice suggest that the cytokines produced during VV infection may assist in the initial recruitment of LCMV-specific memory CD8⁺ T cells into the lung and MLN and that IL-12 enhances IFN- γ production by LCMV-specific memory CD8⁺ T cells.

A. Alteration of LCMV-specific memory CD8 T cell repertoire after VV challenge

To examine whether there was some selectivity to the accumulation of LCMV-specific CD8⁺ T cells in VV-infected LCMV-immune mice, I analyzed the number and proportion of each epitope-specific CD8⁺ T cell population in the total LCMV-specific pool. Figure 4a shows that 12 days after VV infection, there were selective 6- and 80-fold expansions of GP-33- (day 0: 4.0×10^4 cells; day 12: 2.4×10^5 cells) and NP205- (day 0: 3.9×10^3 cells; day 12: 3.1×10^5 cells) specific CD8 T cells, respectively, in the VV-challenged LCMV-immune mouse lungs as compared to the lungs of LCMV-immune mice without VV challenge. There also were 3- to 4-fold increases of three other epitope-specific CD8 T cells. When I analyzed the proportion of each epitope-specific CD8 T cell population within the total LCMV-specific pool (Figure 16), there was a seven-fold selective expansion of the NP205-specific population, retention of the GP33-specific

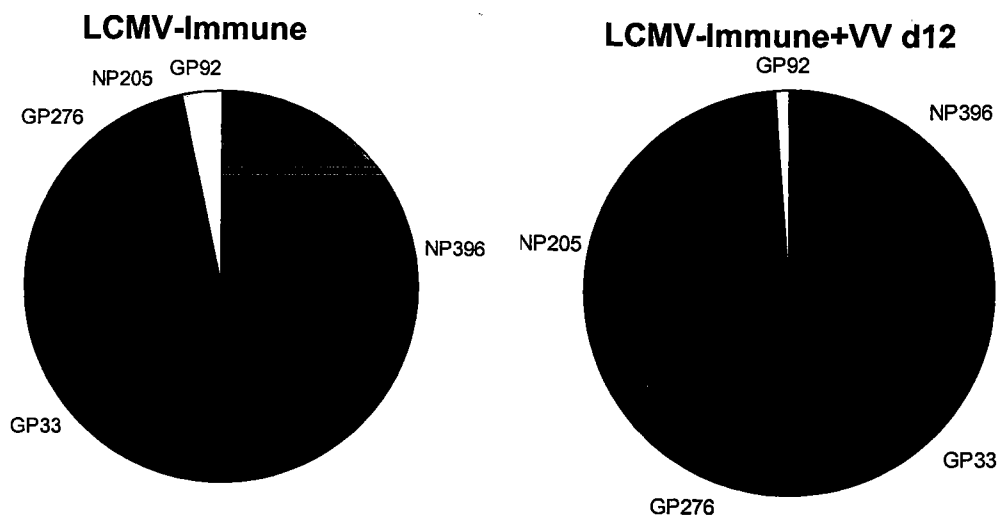


Figure 16. Alteration of LCMV-specific CD8 memory T cell repertoire in VV-infected LCMV-immune mouse lung. This is Figure 6 presented in a new way.

population, but a 50–67% loss of the NP396-, GP276- and GP92-specific populations. Overall, these data indicated that VV infection in an LCMV-immune mouse can markedly alter the LCMV-specific memory T cell hierarchy, with selective expansion of some epitope-specific CD8 T cell populations. This result is suggestive of cross-reactive responses.

It is noteworthy that experiments varied in the relative amount of gp33-specific vs. NP205-specific T cells stimulated. Some experiments showed LCMV-immune mice in which only the NP205 response was significantly amplified, but other experiments showed mice in which the gp33 response was marginally higher than the NP205 response and considerably higher than the normally strong NP396 response. Therefore, upon heterologous VV challenge there was preferential expansion of LCMV-specific memory CD8 T cells with discrete specificities. This selectivity suggests that cross-reactive responses may play a role in this expansion.

B. NP205-cross-reactive VV peptides stimulate LCMV NP205-specific CD8 T cells

Since NP205-specific CD8 T cells were selectively expanded upon VV challenge of LCMV-immune mice, our laboratory pursued a study to search for NP205 cross-reactive VV epitopes. Using DNASIS software to scan the whole VV genome sequence based on two criteria, partial homology to LCMV NP205 and an appropriate MHC K^b binding motif, we found three VV peptides designated as p1, p10 and p24 (Sheridan and Selin, unpublished data). As schematically shown in Figure 17, VV p1 and p24 have about 50% homology to LCMV NP205. They also have H2K^b binding residues at the fifth

LCMV NP205	<u>Y T V K Y P N L</u>
VV p1	<u>Y</u> <u>N</u> <u>S</u> <u>L</u> <u>Y</u> <u>P</u> <u>N</u> <u>V</u>
VV p10	<u>S</u> <u>T</u> <u>L</u> <u>N</u> <u>F</u> <u>N</u> <u>N</u> <u>L</u>
VV p24	<u>A</u> <u>I</u> <u>V</u> <u>N</u> <u>Y</u> <u>A</u> <u>N</u> <u>L</u>

Figure 17. NP205-crossreactive VV peptides. Three VV peptides designated as p1, p10 and p24 were found to have partial 38% to 50% homology to LCMV NP205 and the appropriate MHC K^b binding motif based on scanning of the whole VV genome sequence with the DNAsis program. Homologous residues are shown in bolded letters. They also have the H2K^b binding residue at the fifth Y or F and the eighth L.

Y and the eighth V or L. VV p10 has about 38% homology to LCMV NP205 and H2K^b binding residues at the fifth F and the eighth L. Preliminary experiments by others in the lab showed that LCMV acute infection can induce low numbers of CD8 T cells recognizing VV p1 and p24, whereas VV acute infection can induce p10- and p-24-specific CD8 T cells. In VV-infected LCMV-immune mice, there were increases in those three VV peptide-specific CD8 T cells.

To directly examine whether LCMV-NP205-specific CD8 T cells can be stimulated by VV peptides to produce IFN- γ , I incubated cells with individual VV peptides for 5 hrs and then stained cells with NP205 tetramer or dimer and anti-IFN- γ mAb. As shown in Figure 18, in the resting LCMV-immune mouse lungs, 29% of NP205 dimer⁺ cells synthesized IFN- γ in response to VV p1 stimulation. Twelve days after VV infection, 17% of NP205 dimer⁺ cells were IFN- γ ⁺ in response to VV p1 stimulation. After calculating the cell number, there was actually a 16-fold increase in NP205-specific CD8 T cells recognizing VVp1 in the LCMV-immune lungs 12 days after VV challenge, in addition to a 98-fold increase in NP205-specific CD8 T cells and a 100-fold increase in total CD8 T cells in response to VV p1 (Table 12 Exp. 1). Although 1.1% and 1.3% of CD8 T cells responded to VVp10 and p24 stimulation, respectively, in the resting LCMV-immune lungs, no significant binding of NP205 tetramer was seen in those populations (Figure 18). By day 12 of acute VV infection of LCMV-immune mice, there were 77- and 91-fold increases in CD8 T cells responding to VV p10 and p24 stimulation, respectively, but no significant binding of NP205 tetramer was again seen in those populations (Figure 18 and Table 12 Exp. 1). In the MLN, a 7.7-fold increase in NP205-specific CD8 T cells in response to VVp1 stimulation was also observed in VV-infected LCMV-immune mice (Table 12 Exp. 2). In splenocytes, 43% of

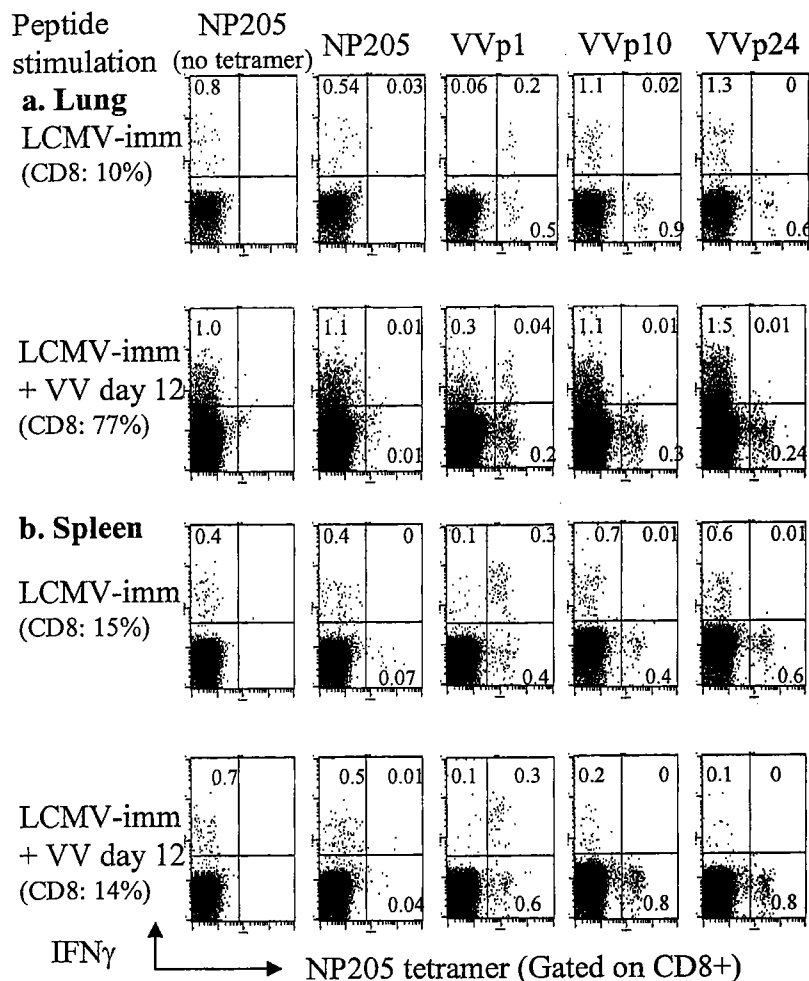


Figure 18. VV peptides cross reactivate LCMV NP205-specific CD8 T cells. LCMV-immune (>12 months after LCMV i.n. infection) mice were challenged i.n. with VV. Leukocytes were isolated from the (a) lungs and (b) spleens, stimulated with individual peptides for 5 hrs and stained with NP205 tetramer and anti-IFN- γ mAb. Cells from groups of three mice each were pooled before staining. The total CD8+ T cell frequencies are given in parentheses. The number in each quadrant is the percentages of CD8+ T cells with a particular staining pattern. The percentage of IFN- γ -producing CD8+ T cells with no peptide stimulation was below 0.01%. Data are representative of three similar experiments, and in two other experiments NP205 dimer was used instead of NP205 tetramer.

Table 12. IFN- γ -producing CD8 T cells in response to VV p1, p10, and p24 stimulation and LCMV NP205-specific CD8 T cells in response to VV p1 stimulation after VV challenge of LCMV-immune mice

Organ	Exp #	Groups	NP205-specific ¹	NP205 ⁺ IFN- γ ² (VVp1 stimulation)	IFN- γ ¹ (VVp1 stimulation)	IFN- γ ¹ (VVp10 stimulation)	IFN- γ ¹ (VVp24 stimulation)
Lung	1 ³	LCMV-imm	9.7 $\times 10^2$	2.4 $\times 10^2$	3.2 $\times 10^2$	1.4 $\times 10^3$	1.6 $\times 10^3$
		LCMV-imm+VV day 12	9.5 $\times 10^4$	3.8 $\times 10^3$	3.2 $\times 10^4$	1.1 $\times 10^5$	1.4 $\times 10^5$
	2 ⁴	LCMV-imm	4.2 $\times 10^3$	5.5 $\times 10^2$	5.5 $\times 10^2$	1.7 $\times 10^2$	2.2 $\times 10^2$
		LCMV-imm+VV day 12	1.8 $\times 10^4$	3.2 $\times 10^3$	5.3 $\times 10^3$	6.6 $\times 10^4$	2.6 $\times 10^4$
	3 ⁴	LCMV-imm	6.0 $\times 10^3$	3.5 $\times 10^2$	4.8 $\times 10^2$	2.8 $\times 10^2$	2.5 $\times 10^3$
MLN	2	LCMV-imm	3.8 $\times 10^4$	3.2 $\times 10^3$	2.2 $\times 10^4$	7.5 $\times 10^4$	7.6 $\times 10^4$
		LCMV-imm+VV day 12	7.3 $\times 10^2$	1.3 $\times 10^2$	1.4 $\times 10^2$	2.6 $\times 10^1$	1.1 $\times 10^1$
	3	LCMV-imm	4.4 $\times 10^3$	1.0 $\times 10^3$	1.0 $\times 10^3$	1.6 $\times 10^3$	1.0 $\times 10^3$
		LCMV-imm+VV day 12	3.9 $\times 10^2$	3.9 $\times 10^1$	1.5 $\times 10^2$	3.5 $\times 10^1$	4.9 $\times 10^1$
Spleen	1	LCMV-imm	3.1 $\times 10^3$	4.3 $\times 10^2$	5.5 $\times 10^2$	1.1 $\times 10^3$	1.7 $\times 10^3$
		LCMV-imm+VV day 12	3.2 $\times 10^4$	2.4 $\times 10^4$	3.2 $\times 10^4$	5.7 $\times 10^4$	4.9 $\times 10^4$
	3	LCMV-imm	4.7 $\times 10^4$	2.0 $\times 10^4$	2.7 $\times 10^4$	1.3 $\times 10^4$	6.7 $\times 10^3$
		LCMV-imm+VV day 12	7.8 $\times 10^4$	3.0 $\times 10^2$	3.0 $\times 10^2$	1.7 $\times 10^1$	1.5 $\times 10^4$
		LCMV-imm+VV day 12	3.3 $\times 10^4$	1.5 $\times 10^3$	2.1 $\times 10^3$	3.4 $\times 10^3$	4.1 $\times 10^3$

1, determined by intracellular IFN- γ staining following indicated individual peptide stimulation; 2, determined by intracellular IFN- γ staining after VVp1 peptide stimulation followed by staining with NP205-specific MHC tetramer or dimer. 3, NP205-specific tetramers were used. 4, NP205-specific dimers were used.

NP205 dimer⁺ cells also were IFN- γ ⁺ in response to VV p1 stimulation. Twelve days after VV infection, 33% of NP205 dimer⁺ cells were IFN- γ ⁺ in response to VV p1 stimulation, although there was no increase in the absolute numbers of both NP205-specific and VVp1-specific CD8 T cells before and after VV challenge (Figure 18 and Table 12). No significant binding of NP205 tetramer was seen in VVp10 and p24-specific CD8 T cells in the spleen (Figure 18). These results suggest that VVp1 can stimulate LCMV NP205 CD8 T cells in a cross-reactive manner, but cross-reactivity with VVp10 and p24 is less defined.

Of note, when cells were stimulated with NP205 peptide for 5 hrs and then stained with NP205 tetramer, IFN- γ ⁺ CD8 T cells responding to NP205 peptide stained with NP205 tetramer to a very low level, presumably because the TCRs on NP205-specific CD8 T cells were down-regulated (Figure 18). The staining patterns with anti-IFN- γ mAb for NP205-specific cells were only slightly different in the absence or presence of NP205 tetramer following NP205 peptide stimulation (Figure 18). Therefore, because of this caveat of the technique used here, the results shown in Figure 18 could be underestimates of cross-reactive cells, arguing for the possibility that VVp10 and p24 are cross-reactive with LCMV NP205-specific memory CD8 T cells. Since VV encodes about 200 proteins, it is highly possible that there are more VV epitopes cross-reactive with other LCMV epitope-specific CD8 T cells.

C. Poly I:C induces up-regulation of CD122 and CD69 on CD8 T cells and a transient increase in LCMV-specific CD8 T cells in LCMV-immune mouse lungs

To assess the role of cytokines in modulation of memory CD8 T cells during heterologous virus infection, I first examined the effect of a cytokine inducer, poly I:C,

on activation and accumulation of LCMV-specific memory CD8 T cells. Memory CD8 T cells have receptors for IL-15, a cytokine that regulates their homeostasis (Ku et al., 2000; Sprent et al., 1999). During viral infections, there is an induction of type I IFN, which can then induce IL-15. In turn, IL-15 can enhance the division of memory CD8 T cells (Ku et al., 2000; McNally et al., 2001; Tough et al., 1996). Poly I:C, which mimics viral dsRNA, is a potent inducer of type I IFN and other cytokines such as IL15 (in both type I IFN-dependent and independent manners) (Mattei et al., 2001). To examine how memory T cells respond to cytokines induced by poly I:C, I first assessed the expression of CD122 (IL-15R β and IL-2R β) on CD8 T cells in LCMV-immune mouse lungs upon intranasal poly I:C treatment. As shown in Figure 19a, CD122 was expressed on CD8 T cells in resting LCMV-immune mouse lungs. By day 3 poly I:C treatment, there was an up-regulation of CD122 expression on the total CD8 T cell population in the LCMV-immune mouse lung. A slight up-regulation of CD122 was also observed on CD8 T cells from the lungs of VV-infected LCMV-immune mice by day 3 postinfection. Similarly, poly I:C-induced up-regulation of CD122 was also observed on CD8 T cells in the MLN of LCMV-immune mice at day 3 post treatment (not shown). It is not clear whether 100 μ g poly I:C induces equivalent levels of cytokines to the amount of cytokines induced by VV infection. Nonetheless, these results suggest that both poly I:C and VV infection could lead to up-regulation of CD122 on CD8 T cells in LCMV-immune mouse lungs, enabling these CD8 T cells to respond to IL-15 induced in those mice.

I also examined CD69 expression on CD8 T cells in LCMV-immune mouse lungs after poly I:C treatment. As shown in Figure 19b, in resting LCMV-immune mouse lungs a very low level of CD69 was expressed on CD8 T cells. A dramatic up-regulation of

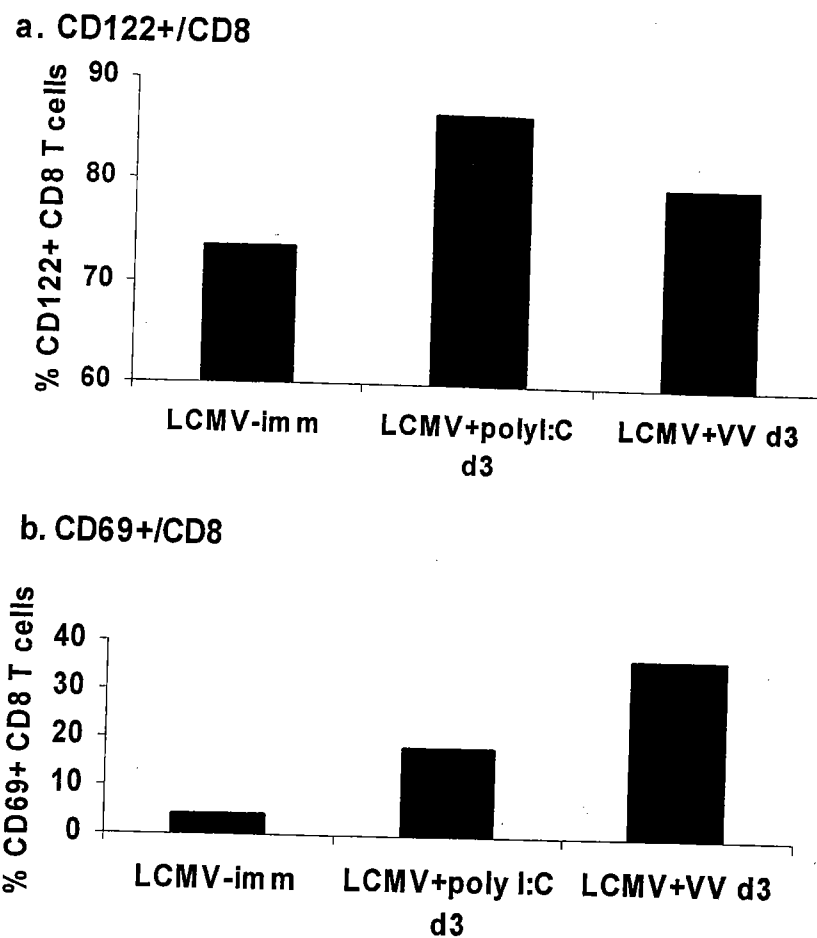


Figure 19. Poly I:C-induced up-regulation of CD122 and CD69 on CD8 T cells in LCMV-immune mouse lungs. LCMV-immune (>12 months after LCMV i.n. infection) mice were inoculated i.n. with 100 μ g poly I:C. Leukocytes were isolated from the lungs, and stained with anti-CD8, anti-CD122 and anti-CD69 mAbs.

CD69 was observed on CD8 T cells from the lungs of VV-infected LCMV-immune mice 3 days after VV infection (Figure 19b), as also shown in Chapter III. However, three days after poly I:C treatment, there was only a slight increase in CD69 expression on the total CD8 T cell population in the LCMV-immune mouse lung (Figure 19b). Type I IFN has been known to up-regulate MHC class I complex expression on APCs (Bukowski and Welsh, 1985; Rhodes et al., 1986); thus it is possible that poly I:C-induced cytokines such as type I IFN might up-regulate MHC class I charged with endogenous self-ligands for TCRs on APCs. These increased endogenous self-ligands could trigger TCRs on T cells, leading to CD69 expression.

Summing intracellular IFN- γ responses to 5 individual LCMV-specific epitopes, there were 9- and 18-fold increases in LCMV-specific CD8 T cells in the lung and MLN of LCMV-immune mice, respectively, 4 days after poly I:C treatment, as compared to resting LCMV-immune mice (Figure 20). However, by 12 days after treatment, there was no further increase but instead a decrease in LCMV-specific CD8 T cells as compared to the cell number at day 4 post treatment (Figure 20). This contrasts with the VV infection of LCMV-immune mice, where there were significant increases in the numbers of LCMV-specific T cells at later time points (Figure 5). Therefore, the poly I:C-induced increase of LCMV-specific CD8 T cells was transient.

D. Poly I:C causes no alteration of LCMV-specific CD8 T cell repertoire and no *in vivo* IFN- γ production in LCMV-immune mice

To examine whether there was a poly I:C-induced nonspecific bystander accumulation of all LCMV-specific CD8⁺ T cells or whether there was some selectivity to

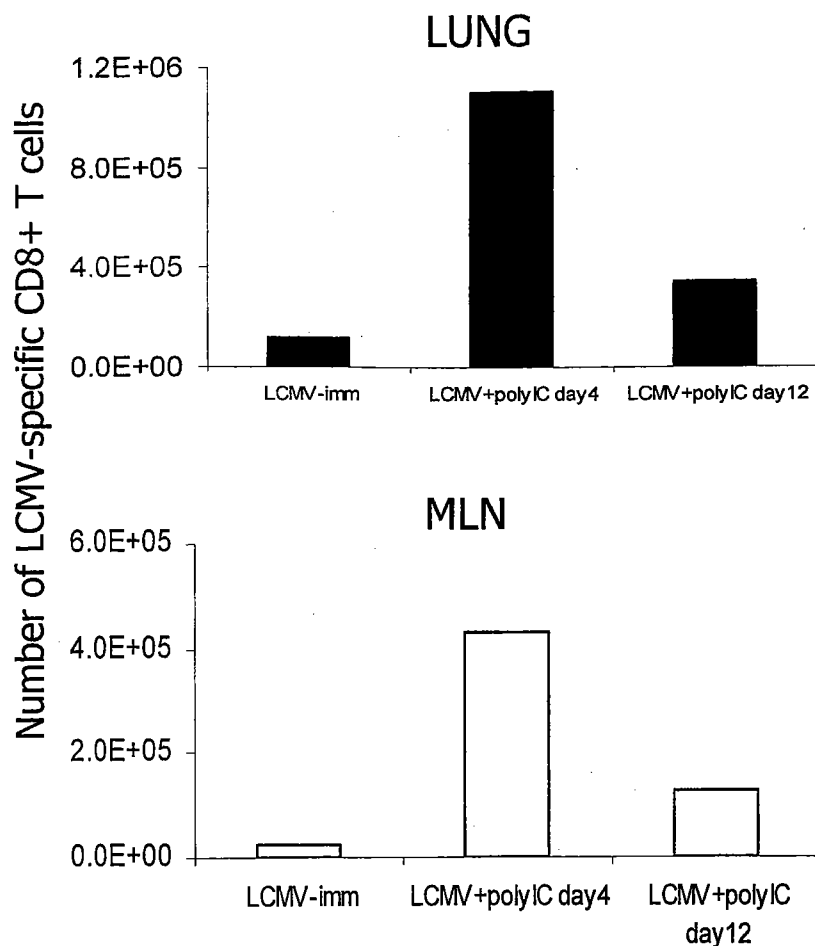


Figure 20. Poly I:C-induced transient increase in LCMV-specific CD8 T cells in LCMV-immune mice.

LCMV-immune (>12 months after LCMV i.n. infection) mice were inoculated i.n. with 100 μ g poly I:C. Lymphocytes were isolated from the (a) lungs and (b) MLNs, stimulated with 5 individual LCMV peptides and stained for intracellular IFN- γ . Lymphocytes from groups of three mice each were pooled before staining. The sum of the total numbers for each of five LCMV epitopes are shown in y-axis. Data are representative of two similar experiments.

this accumulation, I analyzed the proportion each epitope-specific CD8⁺ T cell population represented in the total LCMV-specific pool (Figure 21). In both the lung and MLN, there were no significant alterations of the LCMV-specific CD8 T cell repertoire by day 4 and day 12-post treatment with poly I:C, suggesting that there was a poly I:C-induced nonspecific bystander accumulation of all LCMV-specific CD8⁺ T cells that was equal. This contrasts with the VV-induced alteration of LCMV-specific CD8 T cell repertoire observed in LCMV-immune mouse lungs and MLN at day 12 after VV challenge (Figure 6).

To examine if poly I:C can cause a functional activation of memory T cells in terms of *in vivo* IFN- γ production, I examined *in vivo* intracellular IFN- γ production by memory T cells 3 days after poly I:C treatment. As shown in Table 13, no *in vivo* IFN- γ production was found by LCMV epitope-specific CD8 T cells in poly I:C-treated LCMV-immune mice. This contrasted with CD8 T cells specific for 4 LCMV epitopes from VV-infected LCMV-immune mouse lungs, which accounted for 59% (6.5/11.0) of IFN- γ -producing CD8 T cells in the lung. These results imply that the selective accumulation and alteration as well as functional activation of LCMV-specific CD8 T cells in the lung of VV-infected LCMV-immune mice were not simply due to a non-specific bystander process caused by VV-induced cytokines.

E. Frequency and repertoire of LCMV-specific CD8 T cells in IL-12KO LCMV-immune mouse lungs

VV induces a potent IL-12 response (Slezak et al., 2000). I decided to examine the role of IL-12 in activation of CD8 T cells in VV-infected LCMV-immune mouse lungs,

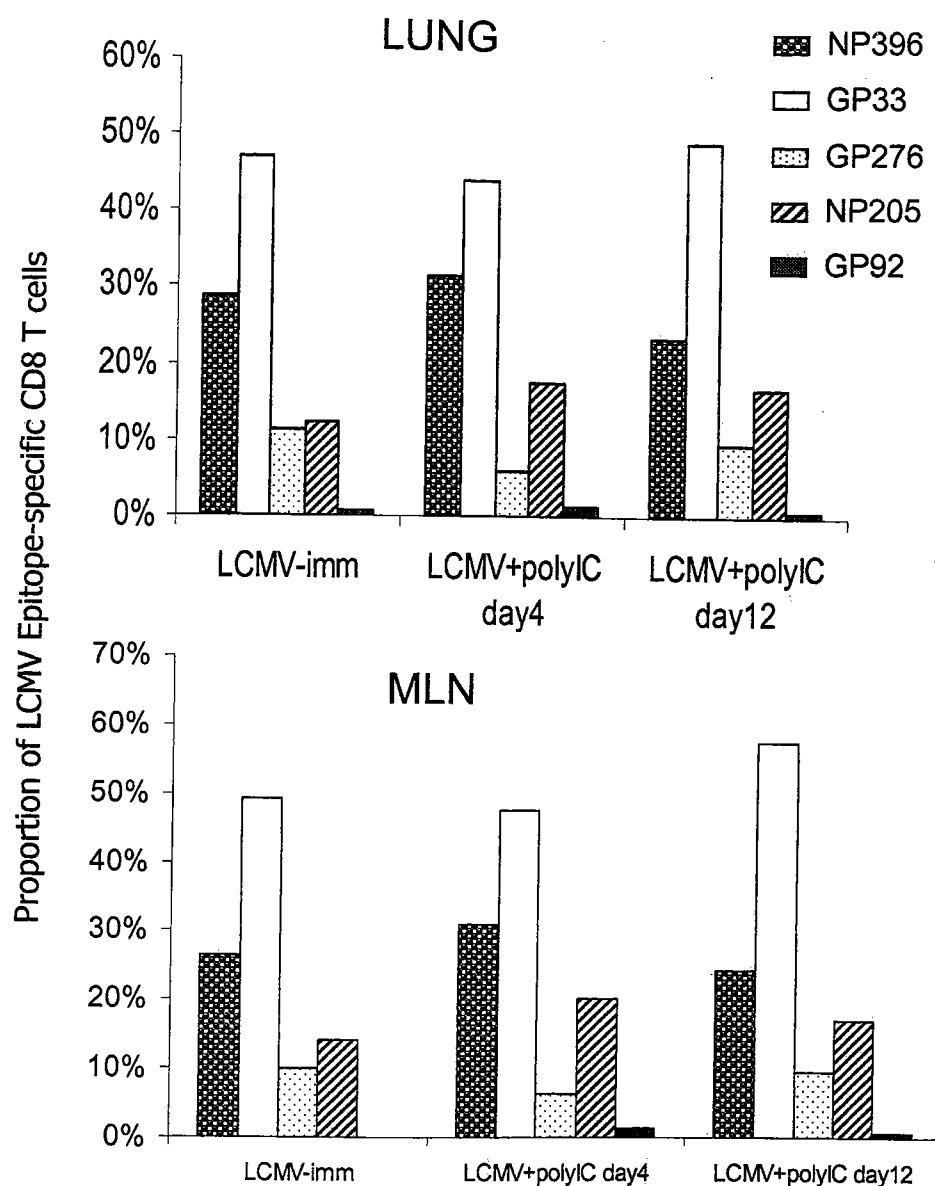


Figure 21. Poly I:C causes no alteration of LCMV-specific CD8 T cell repertoire in LCMV-immune mice. Data were derived from intracellular the IFN- γ staining analysis in Figure 20 a and b with five known LCMV-specific epitopes and are representative of two similar experiments.

Table 13. No *in vivo* IFN- γ production by LCMV-specific CD8 T cells
in poly I:C-treated LCMV-immune mice

Specificity of T cells	% Dimer ⁺ IFN- γ ⁺ cells out of total CD8 ⁺ T cells	
	LCMV-imm + poly I:C	LCMV-imm + VV
	day3	day3
No dimer	0.1	11.0
NP396	0	3.2
GP33	0	1.1
GP34	0	1.9
NP205	0	0.3
Total dimer+		
IFN- γ ⁺ cells	0	6.5

using IL-12KO mice. I observed a similar frequency and repertoire of LCMV-specific CD8 T cells in resting IL-12KO LCMV-immune mouse lungs as compared to the wild-type mice (Figure 22a). Similar results were obtained in the spleens of resting IL-12KO LCMV-immune mice as compared to the wild-type controls (LCMV-immune IL-12KO: 2.0×10^6 cells/spleen; LCMV-immune WT: 1.7×10^6 cells/spleen). In the MLN, there was no significant difference in the frequency of LCMV-specific memory CD8 T cells between the IL-12KO and wild-type LCMV-immune mice. However, the GP33-specific memory CD8 T cell population was no longer predominant in the MLN of resting IL-12KO LCMV-immune mice (WT: $59 \pm 10\%$; IL-12KO: $46 \pm 13\%$; $23\% \downarrow$, $p=0.02$, $n = 3$ experiments). This phenomenon contrasts with GP-33 dominance in the MLN of resting wild-type LCMV-immune mice (Figure 22b). Nonetheless, a relatively normal LCMV-specific memory CD8 T cell pool was detected in the resting LCMV-immune IL-12KO mice.

F. Up-regulation of CD69 and CD44 but not CD122 on CD8 T cells in LCMV-immune IL-12KO mice

To examine how memory T cells respond to VV infection in IL-12KO mice, I assessed CD69 and CD44 expression. As shown in Figure 23, three days after VV challenge, there was a dramatic increase in CD69 expression and a moderate up-regulation of CD44 expression on the total CD8 T cell populations from the lung of both LCMV-immune WT and IL-12KO mice.

However, the expression of CD122 (one subunit of IL-15R and IL-2R) on CD8 T cells was different in LCMV-immune WT and IL-12KO mouse lungs upon VV challenge. It was noted that in both groups, all CD122+ CD8 T cells were CD44+ (not shown). As

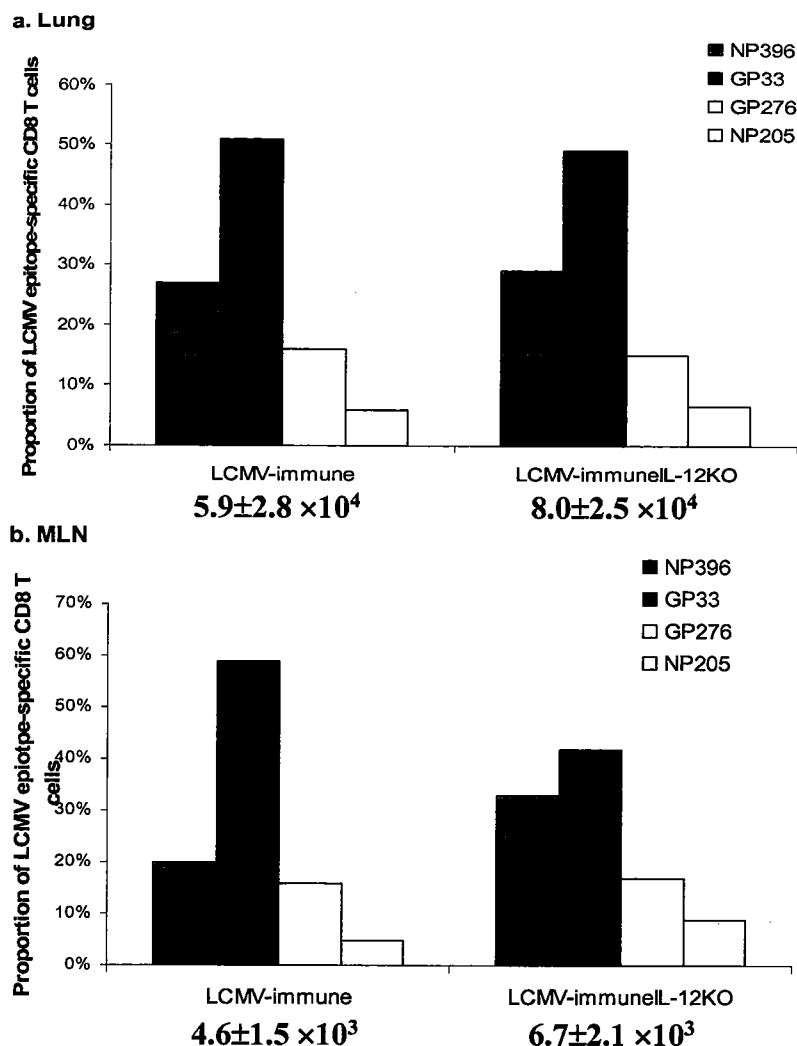


Figure 22. Frequency and repertoire of LCMV-specific memory CD8 T cells in the lung (a) and MLN (b) of resting LCMV-immune IL-12KO mice. Data were derived from intracellular IFN- γ staining analysis with four known LCMV-specific epitopes and are representative of three similar experiments. The numbers below the bar graphs were the sums of the numbers of 4 individual LCMV epitope-specific CD8 T cell populations.

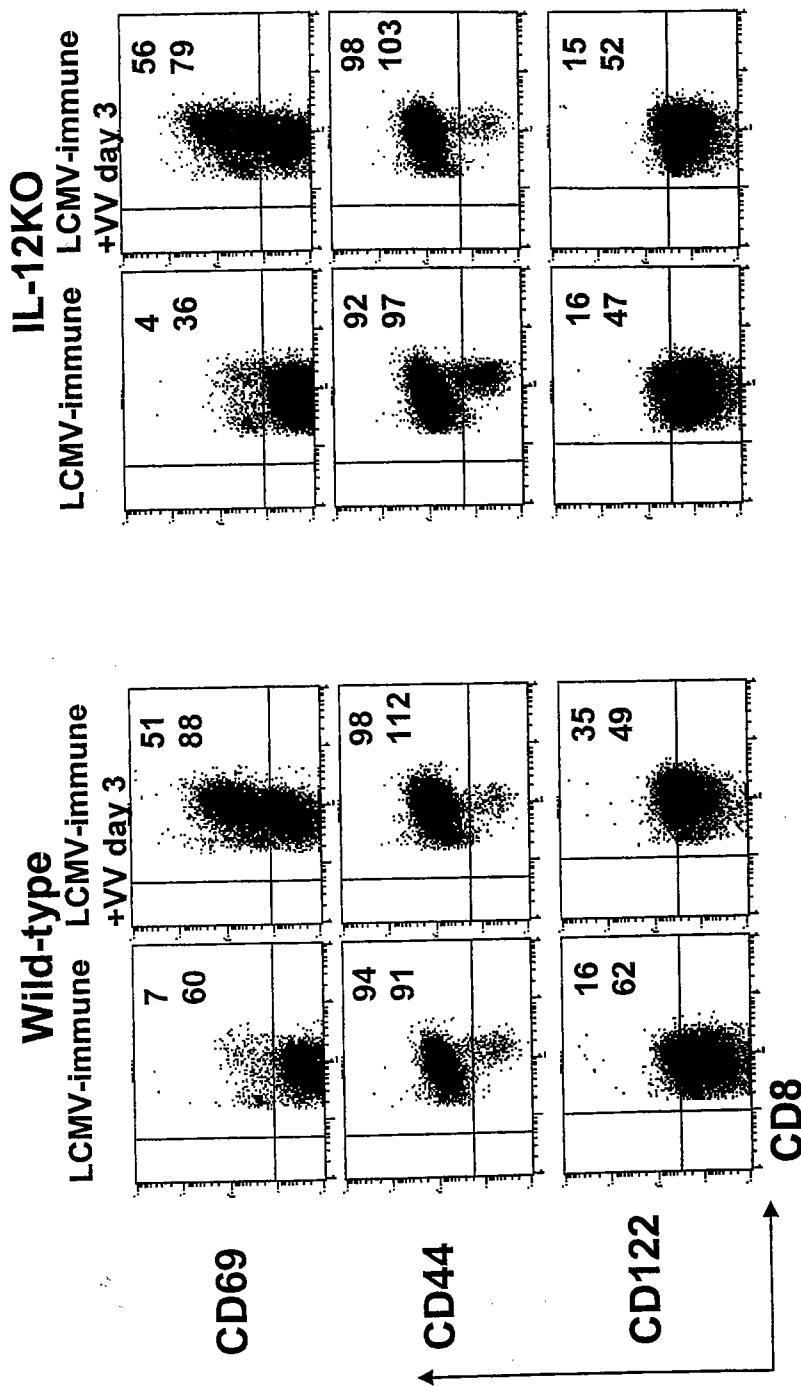


Figure 23. Phenotypic changes on CD8 T cells in VV-infected LCMV-immune IL-12KO mouse lungs. LCMV-immune WT and IL-12 mice were challenged i.n. with VV. Pooled leukocytes were isolated from the lungs of three mice, and stained with anti-CD8, anti-CD69 (a), anti-CD44 (b) and anti-CD122(c) mAbs. Lymphocytes were gated on CD8+. The numbers in the right upper quadrants are the percentages (top) and mean fluorescent intensity (bottom) of CD8+ T cells stained for the indicated markers.

shown in Figure 23, by day 3 VV postinfection, there was a 2-fold increase in the percentage of CD122⁺ CD8 T cells in LCMV-immune WT mouse lung. This was not observed on CD8 T cells from the lungs of VV-infected LCMV-immune IL-12KO mice. Similar patterns of phenotypic changes were observed on CD8 T cells in the MLN of LCMV-immune WT and IL-12KO mice at day 3 post VV infection (not shown). It is not clear why the up-regulation of CD122 expression on CD8 T cells early in VV infections was impaired in the LCMV-immune IL-12KO mice. I postulate that IL-12 might play a role in up-regulation of CD122 expression on CD8 T cells; then, IL-15 secreted by APCs or IL-2 produced by CD4 cells could modulate functions of these CD8 T cells.

G. Accumulation of LCMV-specific CD8 T cells in VV-infected LCMV-immune IL-12KO mice.

To examine if IL-12 plays a role in accumulation of LCMV-specific CD8 T cells in VV-infected LCMV-immune mice, I used intracellular IFN- γ staining to quantify LCMV-specific CD8 T cells at day 0, day 7 and day 12 post VV infection. Data were analyzed by calculating the ratios of the numbers of LCMV-specific CD8 T cells in the wild-type mice versus IL-12KO mice at each time point after VV challenge (Figure 24). In resting immune mice, there were a slightly lower number of LCMV-specific CD8 T cells in the lung, and MLN of wild-type mice than those in IL-12KO mice. Seven days after VV challenge, the numbers of LCMV-specific CD8 T cells in the lung and MLN of wild-type mice were 2.1- and 1.8-fold higher than those in the lung and MLN of IL-12KO mice, respectively. By day 12 post VV infection, the numbers of LCMV-specific CD8 T cells in the MLN of wild-type mice were 3.8-fold higher than those in the MLN of IL-12KO mice.

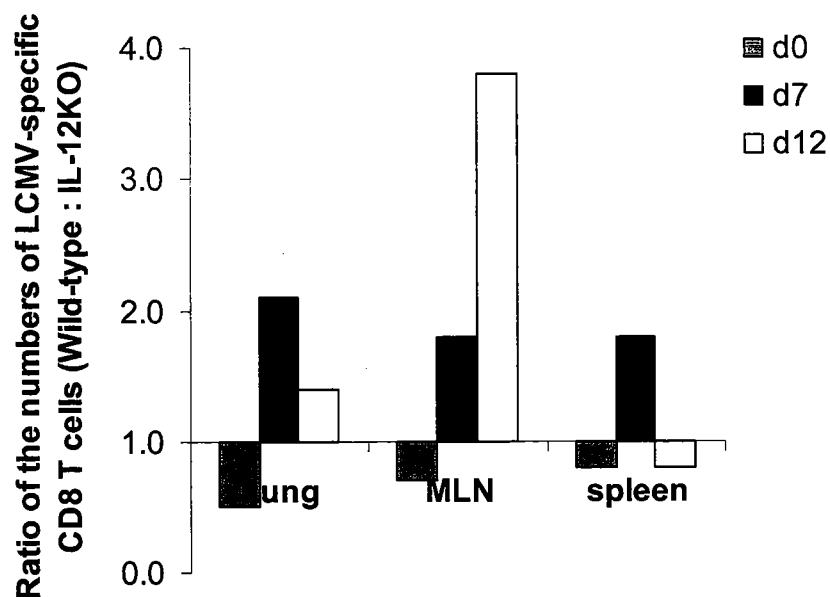


Figure 24. Accumulation of LCMV-specific CD8 T cells in the VV-infected LCMV-immune IL-12KO mice. Data represent the means of the ratios of the numbers of LCMV-specific CD8 T cells in the wild-type mice and IL-12KO mice after VV challenge from three similar experiments (Lung and MLN).

These results suggest that a decreased accumulation of LCMV-specific CD8 T cells occurred in both the lung (day 7) and MLN (day 12) of LCMV-immune IL-12KO mice as compared to the WT controls upon VV infection.

H. The role of IL-12 in IFN- γ production by LCMV-specific CD8 T cells upon antigenic stimulation

When I examined *in vivo* IFN- γ production by LCMV-specific CD8 T cells in VV-infected LCMV-immune IL-12KO mouse lungs using the modified intracellular IFN- γ assay, I found that there was a remarkable 6.25-fold reduction in *in vivo* IFN- γ production by LCMV-specific CD8 T cells in VV-infected LCMV-immune IL-12KO mouse lungs as compared to the WT controls 3 days after VV challenge (Figure 25). Only 6.5% of the LCMV-specific CD8 T cells were producing IFN- γ in VV-infected LCMV-immune IL-12KO mice, while 42% of the LCMV-specific CD8 T cells were producing IFN- γ in VV-infected LCMV-immune WT mice. Therefore, IL-12 played a role in the functional activation of LCMV-specific CD8 T cells to produce IFN- γ *in vivo* during heterologous VV infection.

To further examine whether the IL-12-mediated enhancement of IFN- γ production by LCMV-specific memory CD8 T cells is dependent on TCR triggering, leukocytes from the lung, MLN and spleen of LCMV-immune mice were stimulated with LCMV peptides in the absence or presence of mouse recombinant IL-12 *in vitro* for 5 hrs. Cells were then analyzed by intracellular IFN- γ assays. As shown in Figure 26 and Table 14 from one of two representative experiments, no IFN- γ production by memory CD8 T cells from LCMV-immune mice was observed without cognate peptide stimulation

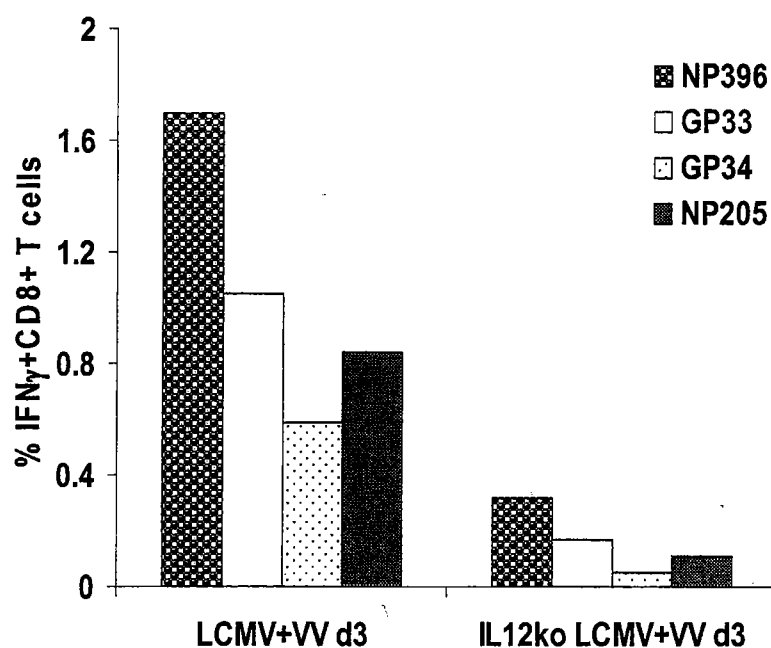


Figure 25. Reduced *in vivo* IFN- γ production by LCMV-specific CD8 T cells in VV-infected LCMV-immune IL-12KO mouse lungs. Mice were infected as indicated. Lymphocytes isolated from mouse lungs were immediately placed at 4 °C and stained with anti-CD8 mAb, 4 of 6 potential LCMV epitope-specific MHC dimers (NP205, NP396, GP33 and GP34) for which we had functioning dimers and anti-IFN- γ mAb without any stimulation *in vitro*. The data presented in this figure have been gated on CD8+ T cells. Y-axis indicates the percentage of CD8 T cells stained with the indicated MHC dimer and intracellular IFN- γ .

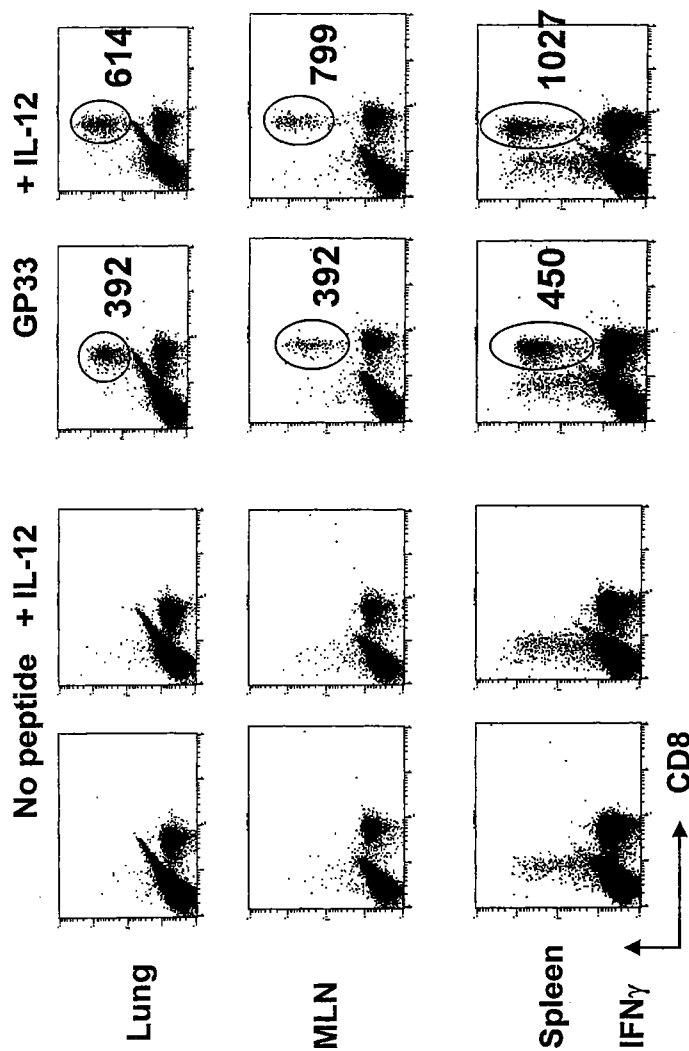


Figure 26. *In vitro* IL-12 treatment enhances peptide induced IFN- γ production by LCMV-specific CD8 T cells from LCMV-immune mice. Leukocytes from the lung, MLN and spleen of LCMV-immune mice were stimulated with 5 individual LCMV epitope-specific peptides in the absence or presence of mouse recombinant IL-12 (125 ng/ml) for 5 hrs. Cells were then analyzed by intracellular IFN- γ assays. The number in each panel is the mean fluorescent intensity (MFI) for IFN- γ staining.

Table 14. *In vitro* IL-12 treatment enhances peptide induced IFN- γ production by LCMV-specific CD8 T cells from LCMV-immune mice

	Lung				MLN				Spleen			
	-IL-12		+IL-12		-IL-12		+IL-12		-IL-12		+IL-12	
No peptide	% ¹	MFI ²	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
NP396	0	0	0	0	0	0	0	0	0	0	0	0
GP33	8.5	383	9.0	529	2.9	397	3.1	738	4.5	496	4.5	944
GP276	13.9	392	14.5	614	5.4	392	6.2	799	8.7	450	8.9	1027
NP205	1.4	339	1.6	508	0.4	314	0.5	569	1.1	417	1.3	825
GP92	2.1	410	2.8	604	1.3	435	1.3	625	1.6	496	1.6	1021
	0.2	378	0.3	333	0.1	311	0.2	215	0.2	406	0.3	500

Leukocytes from the lung, MLN and spleen of LCMV-immune mice were stimulated with 5 individual LCMV epitope-specific peptides in the absence or presence of mouse recombinant IL-12 (125 ng/ml) for 5 hrs. Cells were then analyzed by intracellular IFN- γ assays. **1**, percentage of IFN- γ ⁺ cells out of the total CD8⁺ population specific for each epitope; **2**, MFI, mean fluorescent intensity of bound anti-IFN- γ Ab.

in the presence of exogenous IL-12. If cells were stimulated with individual LCMV epitope-specific peptides, there was a 2- to 3-fold increase in IFN- γ mean fluorescent intensity (MFI) for each epitope-specific CD8 T cell population after addition of exogenous IL-12, even though the frequency of IFN- γ producing cells specific for each LCMV epitope did not significantly increase, suggesting that IL-12 could enhance IFN- γ production by LCMV-specific memory CD8 T cells, but only after their TCRs were triggered by peptide stimulation.

I. Brief summary to Chapter IV

In this chapter, I have shown that upon heterologous VV challenge, there are preferential expansions of LCMV-specific memory CD8 T cells with discrete specificities. This selectivity suggested that cross-reactive responses played a role in this expansion. Moreover, I have shown that VV peptide p1, which is partially homologous to LCMV NP 205, stimulated LCMV-NP205-specific CD8 T cells, suggesting that NP205 may be a cross-reactive epitope. I have provided evidence that poly I:C treatment of LCMV-immune mice results in a transient increase but no repertoire alteration of LCMV-epitope-specific CD8 T cells. These cells do not produce IFN- γ *in vivo*. Finally, VV challenge of LCMV-immune IL-12KO mice results in activation and slightly decreased accumulation of LCMV-specific CD8 T cells. Here, there is a dramatic reduction of *in vivo* IFN- γ production by LCMV-specific CD8 T cells in the lung of LCMV-immune IL-12KO mice. Overall, the cytokines and, possibly, chemokines produced during VV infection may assist in the initial recruitment of LCMV-specific memory CD8⁺ T cells into the lung

and MLN. However, cross-reactive T cell responses most likely participated in their activation and selective expansion in the lung and MLN upon VV challenge.

CHAPTER V

THE SEQUENCE OF INFECTIONS WITH DIFFERENT VIRUSES DETERMINES THE PATTERN OF ANTIVIRAL IMMUNITY AND IMMUNOPATHOLOGY IN THE LUNG

Prior heterologous immunity to a virus can alter the course of disease caused by a second virus. In the previous two chapters, a model of the respiratory VV infection of LCMV-immune mice was examined. In this chapter, I will demonstrate some new insights on heterologous immunity using other virus combinations. Using four unrelated viruses (LCMV, VV, influenza A virus and MCMV), I took two different approaches. First, I examined the effect of immunity to three different viruses, LCMV, MCMV and influenza A virus had on the outcome of acute VV infection. Second, I examined the effect of immunity to one virus, influenza A virus, had on the acute responses to LCMV, MCMV and VV. I focused my assessment of disease outcome on three major features: virus titers, cytokine profiles and lung immunopathology. These studies demonstrate that the specific sequence of infections with different viruses controls the outcome of disease by influencing viral clearance, early cytokine responses and immunopathology.

A. Respiratory infection of C57BL/6 mice with LCMV, influenza A virus or MCMV

I first examined the kinetics of viral clearance after an i.n. inoculation of naïve C57BL/6 mice with LCMV, influenza A virus or MCMV. As shown previously, LCMV was cleared in the lungs and spleens (Table 2). During an acute influenza A virus infection,

influenza A virus could be detected in the infected-mouse lungs by plaque assays (Table 2). By day 10 postinfection, influenza A virus was no longer detectable in the lung. These results were comparable to other studies (Doherty et al., 1992), and influenza A virus-immune mice remained healthy during the rest of their life time unless they were subsequently infected with pathogens.

When mice were immunized with a low dose of MCMV (400 PFU), there was no discernible clinical illness during the course of the infection. MCMV titers in the lung were not examined. When mice were challenged with a much higher dose of MCMV (1×10^5 PFU), MCMV PFU were detected in the lung (Table 16). In mice given 10^4 PFU of MCMV intranasally, the course of virus replication was virtually identical to that noted with 100 PFU of virus (Jordan, 1978). In that study, peak titers were reached in the lungs on day 7 (5×10^5 PFU/lung) and gradually declined over the next 2 weeks.

Therefore, during an respiratory infection of C57BL/6 mice with these selected doses of LCMV, influenza A virus, or MCMV, these viruses were cleared from the lung, and infections with these viruses could be used to study the effect of prior immunity to one virus on immunity against a second unrelated virus.

B. The sequence of heterologous virus infections determines whether viral clearance is enhanced or impeded

The effects of immunity to LCMV, MCMV, or influenza A virus on VV infection. Adult C57BL/6 mice were intranasally immunized with either LCMV, MCMV or influenza A virus. More than 6 weeks after the acute response had resolved, and when the host was in a resting immune state, the mice were challenged with a second virus,

Table 15. Previous heterologous viral infections protect against VV

Immunizing virus	Challenge virus ^a	Days after challenge	Virus Titer (log ₁₀ PFU/lung) ^b	p value (Immune v.s. Control ^c)
Control ^d	VV (3)	6	6.2 ± 0.3	
LCMV ^d			5.5 ± 0.4	P= 0.03
Control	VV (1)	5	7.5 ± 0.1	
MCMV			7.0 ± 0.1	p = 0.004
Control	VV (2)	5	6.2 ± 0.1	
Flu ^e			5.4 ± 0.4	p = 0.1

The infection of mice was done as described in Materials and Methods. a, The number in parentheses represents the number of experiments that were done. b. Data are presented as mean ± SEM log₁₀PFU/lung from a representative experiment. There were three to five mice per treatment group. c. Statistically significant difference between the control and immunized groups of mice (t-test). d. LCMV-immune mice (**12 weeks after LCMV infection**) acutely infected with VV were NK cell-depleted by i.p. injection of 100 µl (200 µg) of anti-NK1.1 (clone PK136) on day 0 and 4 after infection. e, Flu-immune mice were challenged with VV **6 weeks after initial infection** with influenza virus.

Table 16. Prior immunity to Flu can enhance or impede viral clearance

Immunizing virus	Challenge virus ^a	Days after challenge	Virus Titer (log ₁₀ PFU/lung) ^b	P value (Immune vs. Control ^c)
Control	VV (2)	5	7.5 ± 0.1	
Flu ^d			7.1 ± 0.2	↓, P = 0.03
Control	LCMV (2)	5	5.1 ± 0.2	
Flu ^e			5.7 ± 0.1	↑, P < 0.05
Control	LCMV (3)	7	5.5 ± 0.1	
Flu ^e			6.3 ± 0.1	↑, P < 0.05
Control	LCMV (3)	7	5.4 ± 0.4	
Flu ^f			6.0 ± 0.2	↑, P = 0.04
Control	MCMV (7)	7	5.0 ± 0.3	
Flu ^g			5.9 ± 0.1	↑, P = 0.02
Control	MCMV (7)	7	4.7 ± 0.4	
Flu ^h			5.5 ± 0.3	↑, P = 0.03

The infection of mice was done as described in Materials and Methods. a, The number in parentheses represents the number of experiments that were done. b, Data are presented as mean ± SEM log₁₀PFU/lung from a representative experiment. There were three to five mice per treatment group. c, Statistically significant difference between the control and immunized groups of mice (t-test). d, e, f, g, and h, Flu-immune mice were challenged with a second unrelated virus **12, 24, 11, 10, and 6** weeks after initial infection with influenza virus, respectively.

as seen in Table 15 and Table 16, which show viral titration data. Two distinct patterns emerged on how prior infections could influence viral clearance in the lung. The first pattern was that prior immunity to LCMV, MCMV or influenza A virus resulted in 80%, 68% and 84% reductions in VV titers, respectively, in the lung at 5-6 days after VV infection (Table 15). This protective effect of prior immunity lasted for long periods of time after the original virus infection, as mice either 12 (Table 15) or 30 weeks (Control+VV: $6.9 \pm 0.1 \log_{10}\text{PFU/lung}$; LCMV-immune+VV: $6.3 \pm 0.1 \log_{10}\text{PFU/lung}$; 86% ↓, $p < 0.05$, $n = 3$ mice/group) after LCMV infection demonstrated comparable efficiency at resisting VV challenge as did mice either 6 (Table 15) or 12 (Table 16) weeks after influenza A virus infection challenged with VV. Therefore, prior immunity to LCMV, MCMV or influenza A virus provided significant protection against VV infection.

The enhanced replication of LCMV or MCMV in influenza A virus-immune mice. In contrast to the protection against VV in influenza A virus-immune mice, prior immunity to influenza A virus was not always protective. The second pattern I observed was that immunity to influenza A virus resulted in a significant 6.3-fold increase in LCMV and 8-fold-increase in MCMV titers in the lungs when compared to controls 7 days after challenge with LCMV or MCMV (Table 16). Within this same group of influenza A virus-immune mice there was still a 60% decrease in VV titers as compared to controls. Enhanced LCMV titers were seen in the lungs of influenza A virus-immune mice as early as 5 days after challenge, but the increase was not as big as the one observed 7 days after LCMV infection (Table 16). Moreover, significantly increased LCMV titers were also found in the spleens of influenza A virus-immune mice compared with control mice 7 days after LCMV challenge (for instance, control: 3.7 ± 0.3

\log_{10} PFU/spleen; influenza A virus-immune mice: $4.7 \pm 0.5 \log_{10}$ PFU/spleen; 10-fold increase, $p = 0.009$; $n = 4$ mice/group). For MCMV challenge, the kinetics of the increase in virus titer varied between the two stocks of MCMV preparations used. Significant increases in MCMV titers were mostly observed at day 7 postinfection for one stock, while in some cases significant increases in MCMV titers were found at day 9 postinfection for the other stock. The reason for this variation is unclear. These experiments were carried out 6 – 24 weeks after influenza A virus infection (Table 16), indicating that this enhanced LCMV or MCMV replication appeared to be independent of the time after initial immunization with influenza A virus. These studies suggest that exposure to heterologous viruses can be beneficial, resulting in protective immunity, but, depending on the infection sequence, can also result in enhanced virus replication.

In addition to the sequential heterologous viral infections shown in Table 15 and 16, I also assessed virus titers in the lungs of influenza A virus- or MCMV-infected LCMV-immune mice and LCMV- or influenza A virus-infected MCMV-immune mice. In a few pilot experiments no significant changes in challenge virus titers were observed in the lungs of those heterologous virus-immune mice when compared with control mice (Table 17), so no more experiments have been performed with these combinations since then. These studies suggest that immunity between heterologous viruses is a common occurrence, but it is not universal to all virus combinations, as there is a selective effect dependent on the sequence of virus infections.

Table 17. Heterologous immunity between viruses in the lung

Immunizing virus	Challenge virus			
	LCMV	Flu	MCMV	VV
LCMV	ND	- 0.1 (1)	- 0.1 (1)	- 0.9 (4)*
Flu	+ 0.6 (3)*	ND	+ 0.7 (7)*	- 0.6 (2)*
MCMV	+ 0.1 (2)	+ 0.1 (1)	ND	- 0.5 (1)*

Naive mice or mice that were immune to various heterologous viruses were challenged intranasally with these viruses, and the PFU titers were assessed in lungs 5-9 days after infection. Data are presented as average of mean changes of titers in multiple experiments. The number in parentheses represents the number of experiments that were done to derive the mean \log_{10} changes in virus titers. There were three to five mice per treatment group. This table shows the degree of either protective immunity, as determined by the reduction of viral titer, or decreased immunity, as determined by the increase in viral titer, in heterologous virus-immune mice compared with control mice. ND, homologous virus challenges were not done. +, increased titer; -, reduced titer. *, $p < 0.05$.

C. The sequence of heterologous virus infections determines the cytokine profile

I questioned whether prior immunity to heterologous viruses affected the cytokines induced during the subsequent virus infection. Lungs of LCMV-immune mice early after VV challenge or of influenza A virus-immune mice early after VV or MCMV challenge were assessed for their cytokine mRNA levels by RNase protection assay (Figure 27). IFN- γ is a prototypic T_H1 cytokine with antiviral activity. IL-12 is a cytokine with multiple functions, including induction of differentiation of T_H1 T cells. IL-1, IL-6, and TNF- α are three major proinflammatory cytokines inducing neutrophilic infiltration and synthesis of acute phase proteins.

The cytokine profile in VV-infected LCMV-immune mice. Consistent with my early study on VV-infected LCMV-immune mouse lungs, there were 4-fold higher levels of IFN- γ mRNA in LCMV immune mice lungs 3 days after VV infection (Figure 27a). At the same time, there was also a 50% reduction in the mRNA levels of the pro-inflammatory cytokine IL-6 in the LCMV-immune mouse lungs as compared to the controls. Altogether these results suggested that stronger acute inflammatory cytokine responses occurred in the VV-infected control mice as compared to LCMV-immune mice. Previous immunity to LCMV markedly enhanced a Th1 type cytokine response to VV infection but appeared to suppress pro-inflammatory cytokine responses.

The cytokine profile in VV-infected influenza A virus-immune mice. Cytokine profiles upon VV infection of influenza A virus-immune mice mirrored the ones observed in LCMV-immune mice. There was a 60% higher level of IFN- γ mRNA

Figure 27

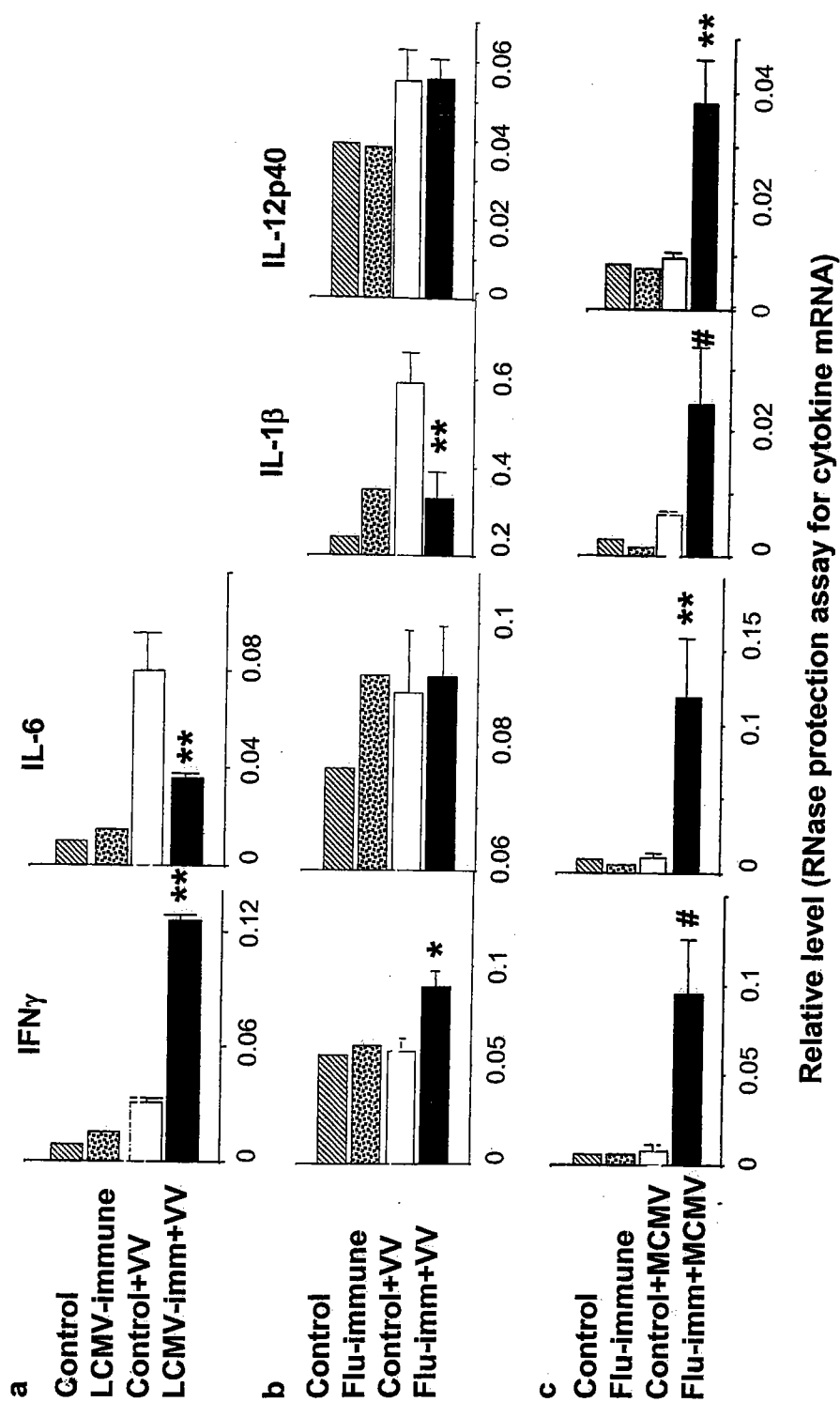


Figure 27. Cytokine profile alteration during heterologous virus infections varies depending on the specific virus infection sequence. Total RNA was isolated from the whole lung tissue. Detection and quantification of a variety of murine cytokine mRNAs were accomplished as outlined in the Methods. There were three mice in virus-challenged immune and control groups and one mouse in the control and immune groups. The results were presented as relative ^{32}P intensities of the signals for individual cytokines, which were normalized to the levels of house-keeping gene expression. **, $p < 0.05$; #, $p = 0.07$; *, $p < 0.1$. Data were representative of 2 or 3 similar experiments. There was little variation among experiments in cytokine signals from the single mouse of the control and immune groups.

expression in the lungs at day 3 after VV infection in influenza A virus-immune mice as compared to controls (Figure 27b). Concomitantly, these same influenza A virus-immune mice showed a 50% reduction in mRNA levels of the pro-inflammatory cytokine IL-1 β , although IL-6 levels remained similar to control mice (Figure 27b). It would appear in situations where prior immunity resulted in protective immunity against VV infection there were enhanced Th1 type responses with increased IFN- γ associated with suppression of some pro-inflammatory responses upon VV challenge.

The cytokine profile in MCMV-infected influenza A virus-immune mice.

When I examined the cytokine profile in influenza A virus-immune mouse lungs challenged with MCMV, where prior immunity appeared to have a negative impact on the second virus infection, I observed that a very different cytokine profile was induced by MCMV as compared to VV. There was a very striking 12-fold increase in IFN- γ mRNA level when compared to control mice 3 days after MCMV challenge (Figure 27c). However, unlike the influenza A virus or LCMV-immune mice challenged with VV, the MCMV-infected influenza A virus-immune mice had significantly increased levels of pro-inflammatory cytokines, including 11-, 4- and 4-fold increases in IL-6, IL-1 β and IL-12p40, respectively (Figure 27c). It is not clear what mechanism accounts for the increased MCMV load in influenza A virus-immune mice. It is possible that the increased virus load is driving the production of these cytokines, or it is also consistent to assume that the presence of these pro-inflammatory cytokines may mediate the enhanced virus replication.

D. Distinctive lung pathology during acute respiratory infection of non-immune mice with LCMV, MCMV, influenza A virus, or VV

I questioned whether it is a general phenomenon that prior infections with heterologous viruses could influence lung pathology on subsequent infections; thus, I needed to document baseline data on lung pathology during acute infection in non-immune mice. Mouse lung tissues were harvested, sectioned and stained with hematoxylin and eosin. Each acute virus infection in naive mice at the selected doses induced a distinct lung pathology, which returned to essentially normal architecture in the resting immune state (Figure 28). Figure 28a shows a naïve mouse lung section, characterized by flat pleura, clean and open bronchioles, clean and open alveoli with very thin septa, as well as normal vasculature. Acute **LCMV and MCMV** infections induced relatively mild lung disease composed predominantly of chronic mononuclear (MN) infiltrates in the interstitium (Figure 28b, c, and Table 18). Acute LCMV infection could induce mild to moderate transient BAL. BAL is not normally visible but can be induced by infection (Figure 28b and Table 18). MCMV only induced BAL at very high doses of infections (Table 18). MCMV also is known to infect endothelial cells (Staczek, 1999) and thus it was not too surprising to observe endothelial activation (Table 18) upon infection with this virus.

Influenza A virus and VV infections at much lower doses of virus than LCMV and MCMV caused much more severe lung damage, with some residual changes in the lung. **Influenza A virus** induced severe acute inflammation with many mixed polymorphonuclear (PMN) and MN cells around airways, in the alveolar spaces and interstitium. This severe acute inflammation frequently developed into consolidation.

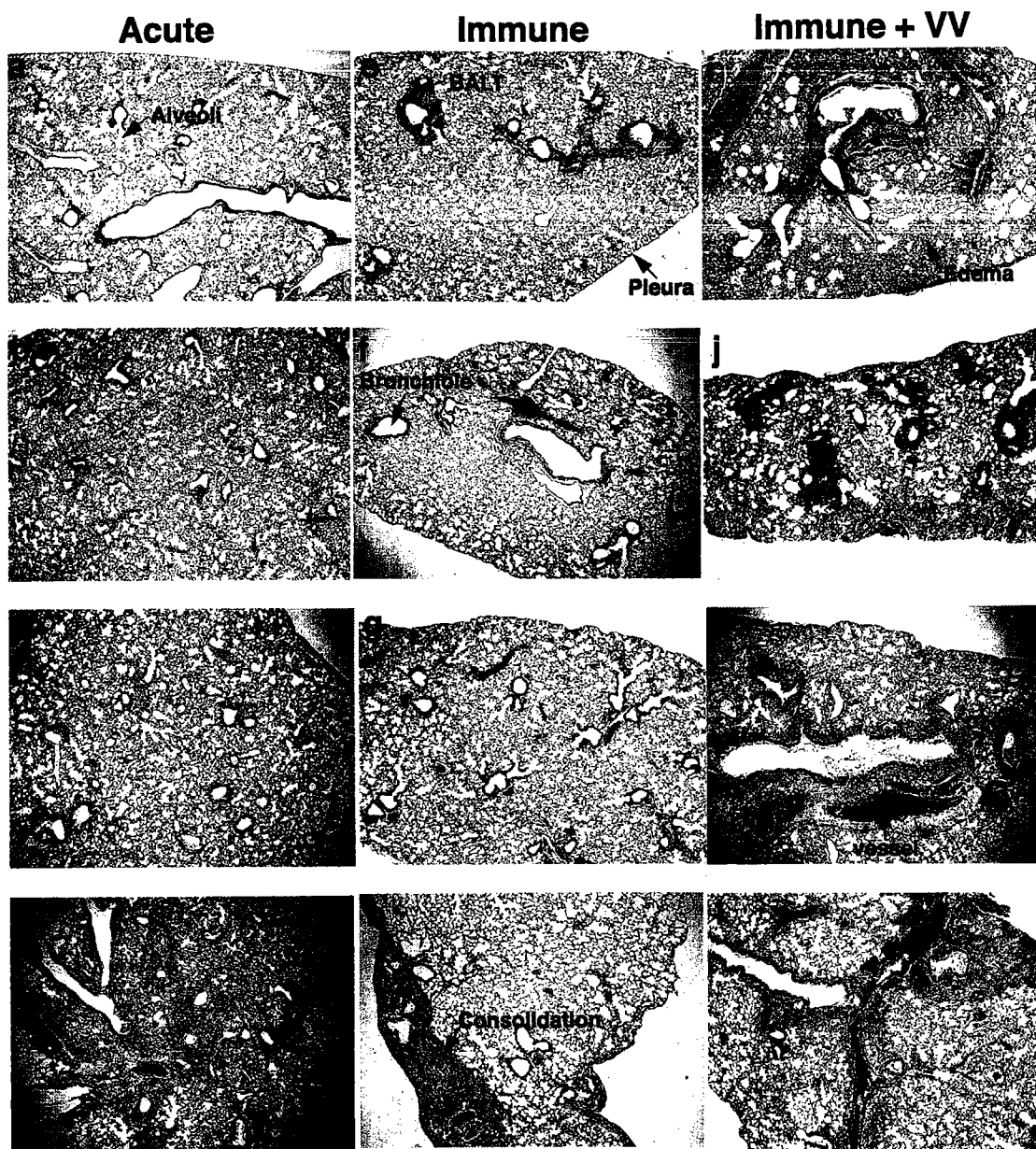


Figure 28. Lung immunopathology varies depending on the infecting virus and can be further dramatically altered by prior immunity to heterologous viruses. Lung sections from mice were stained with H+E. First column: naïve control (a) and single virus acute infection (day 7) (b-LCMV, c-MCMV, d-Flu); second column: single virus immune (e-VV, f-LCMV, g-MCMV, h-Flu); third column: immune mice infected with VV (i-naïve+VV day 7, j-LCMV-immune+VV day 7, k-MCMV-immune+VV day 5, l-Flu-immune+VV day 5). Magnification: 4× of the objective of the digital light-microscope.

Table 18. An acute respiratory infection with 4 different viruses causes distinct immunopathological changes in the lung

Lung Compartments	Pathological Features	Severity grading ^c			
		LCMV (n = 4) ^d	MCMV ^f (n = 6)	Influenza virus (n = 3)	VV (n = 11)
Alveoli	Edema AMI ^a	0.5 (0 - 1) ^e		1.3 (1 - 2) 3	2 (0 - 3)
BALT	Extent of BALT	1.0 (0 - 2)	0.8 (0 - 1)	0.6 (0 - 1)	0.5 (0 - 2)
Airways	Necrotizing bronchiolitis			1 (0 - 2)	1.4 (0 - 3)
	Bronchiolitis		0.2 (0 - 1)	1.6 (0 - 3)	1.5 (0 - 3)
	Peribronchial AMI	0.5 (0 - 1)			1.2 (0 - 3)
	Peribronchial CMI		0.2 (0 - 1)		
	Consolidation			2 (0 - 3)	
Interstitialium	CMI ^b	1.3 (1 - 2)	2.2 (2 - 3)	3	
	AMI			2.6 (2 - 3)	0.5 (0 - 3)
Pleura	CMI	1.3 (1 - 2)	2.2 (2 - 3)	2.3 (1 - 3)	0.2 (0 - 1)
	AMI			2.3 (1 - 3)	
Vasculature	Lymphocytic cuffing and tufting	0.5 (0 - 1)	2.2 (2 - 3)	1	
	Necrotizing vasculitis			0.3 (0 - 1)	0.3 (0 - 2)
	Perivascular CMI	1 (0 - 3)			
	Perivascular AMI			3	0.8 (0 - 3)
	Endothelia activation		1.3 (0 - 2)		
	Edema	0.5 (0 - 1)			0.6 (0 - 3)

Seven to eight days after virus infection the mouse lungs were harvested, sectioned and stained (H+E), as described in Materials and Methods. a, AMI, acute mixed infiltrates with polymorphonuclear and mononuclear cells; b, CMI, chronic mononuclear infiltrates with lymphocytes and macrophages; c, Grading of the pathological changes in the lung: based on the distribution and the severity of disease from 0 to 3 (*blank* or 0, within normal limit; 1, 1-9%, 2, 10-49%, and 3, $\geq 50\%$ involvement of the lung parenchyma). d, the number of mice in each group. e, Data are presented as average of scores from multiple mice per treatment, and the number in parentheses after each average score represents the range of scores from mice challenged with the virus indicated. f, Mice were infected with a *high dose of MCMV* (1×10^5 PFU).

Consolidation in influenza A virus-infected mouse lungs was reported previously in an early study on influenza pneumonia (Loosli et al., 1975). Moderate edema, necrotizing vasculitis and perivascular acute mixed PMN and MN infiltrates were also present in the influenza A virus-infected mouse lungs (Figure 28d and Table 18). After the acute influenza A virus infection resolved, the lung architecture was relatively normal. There were some residual consolidated areas and mild BALT persisting in the influenza A virus-immune mouse lung. Also occasionally, very mild bronchiolization occurred in the influenza A virus-immune mouse lung (Figure 28h and Table 20). Bronchiolization is a damage-repairing process where the alveolar walls are lined by cells resembling, but not necessarily identical with, bronchiolar epithelium. Bronchiolization can be observed after a variety of insults such as respiratory infection, exposure to chemical irritants and carcinogens, and even circulatory disturbances involving the lung (Nettesheim and Szakal, 1972).

During acute **VV** infection, the mouse lungs developed necrotizing bronchiolitis and severe alveolar edema (Figure 28i, 29a and Table 18). This accumulation of extravascular fluid in the air spaces could significantly disturb gas exchange. In addition, the VV-infected mouse lungs mainly showed acute mixed inflammatory infiltrates (AMI) with PMN and MN cells in the peribronchial areas and interstitium, as well as in the perivascular areas, shown at higher magnifications in Figure 29 (Figure 29a, b and Table 18, 20). Enhanced BALT could develop but only late, i.e. greater than 14 days, after VV infection (Table 11). Mild BALT persisted for at least 3 months in the VV-immune mouse lung (Figure 28e).

The findings described above are consistent with previous studies (Jakab et al., 1983; Jordan, 1978; Lemercier et al., 1979; Loosli et al., 1975; Mackenzie et al., 1989; Martinez Peralta et al., 1990; Staczek, 1999; Walker and Murphy, 1987; Yap et al., 1979).

E. Alterations in VV-induced lung pathology is dependent on the specific history of heterologous virus exposure

The effects of immunity to LCMV on VV-induced immunopathology.

When the LCMV-, MCMV- or influenza A virus-immune mice were challenged with VV, the immunopathology varied dramatically not only from the VV-infected controls but also from each other, depending on the specific heterologous virus exposure. During the acute VV infection, lungs from VV-infected control and LCMV-, MCMV- or influenza A virus-immune mice all showed one common pathology, necrotizing bronchiolitis (Figure 29b, e, g and Table 19), which is defined by the presence of necrotic bronchiolar epithelium with PMNs and debris. However, compared to the controls, the lungs of LCMV-immune mice challenged with VV showed very little alveolar edema and had a very prominent lymphocytic response, instead of an acute inflammatory response. Most strikingly, the LCMV-immune mouse lungs had very prominent BALT as early as 5 and 7 days after VV infection (Figure 28j, 29c, d and Table 19). Also, prominent chronic MN infiltration (CMI) with lymphocytes and macrophages was present in the interstitium and pleura of almost all the LCMV-immune mouse lungs. In addition, the LCMV-immune lungs had perivascular lymphocytic cuffing, as defined by a significant lymphocyte accumulation around the vessels (Figure 28j, 29c, d and Table 19). Interestingly, by day

Figure 29

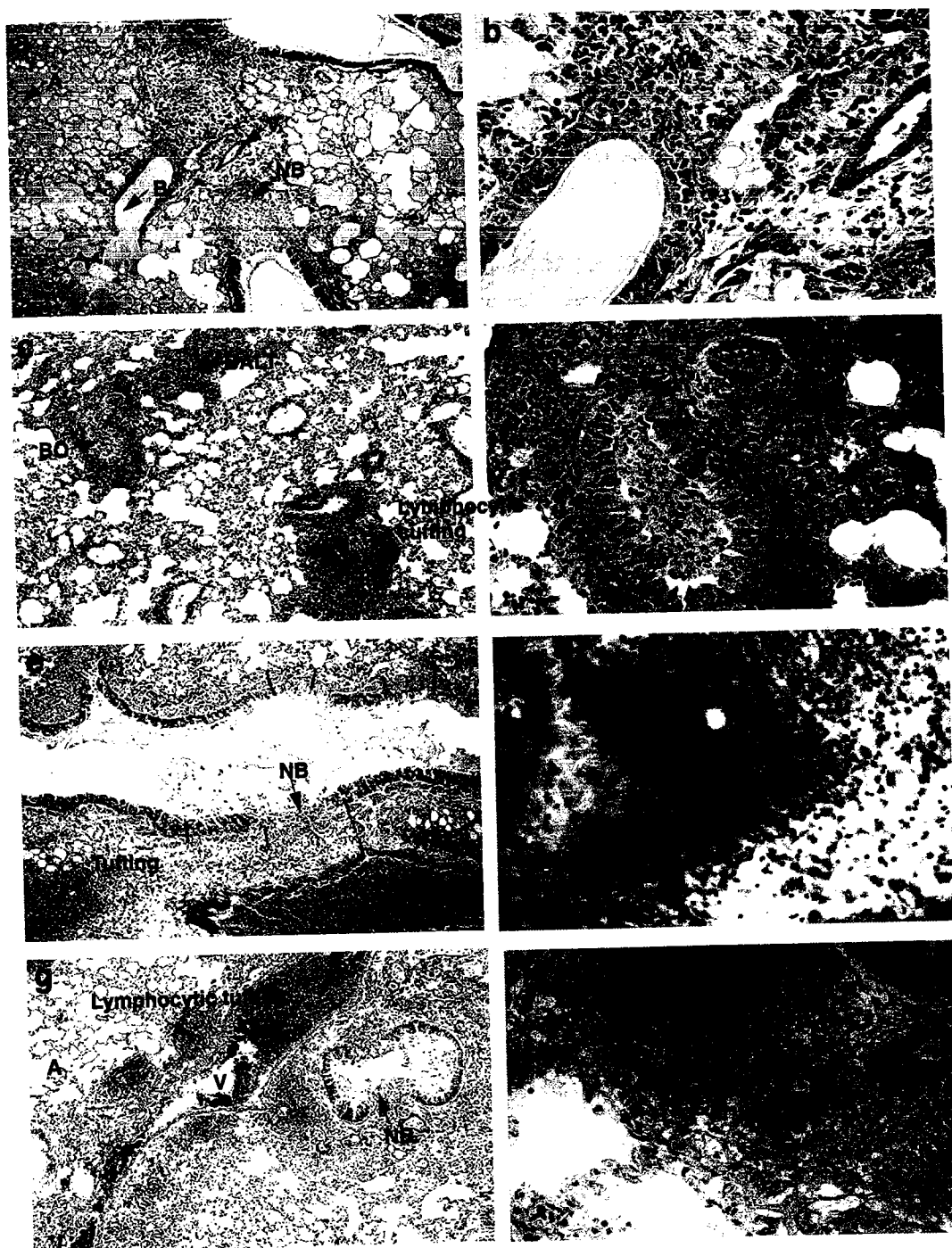


Figure 29. Patterns of chronic mononuclear infiltrates in VV-infected mouse lungs vary depending on prior heterologous virus exposure. Higher magnifications (10×: a, c, e, and g; 40×: b, d, f, and h) of the lung sections shown in panel i, j, k and l of Figure 28 demonstrated in more detail the alteration in lung pathology of naïve mice (a, b) as compared to LCMV-immune (c, d), MCMV-immune (e, f) and Flu-immune (g, h) upon VV challenge. A: alveoli; B: bronchiole; V: vessel; AMI, acute mixed inflammation with PMN and MN infiltrates; BO, bronchiolitis obliterans; NB, Necrotizing bronchiolitis.

Table 19. Prior heterologous virus exposure induces variations in chronic mononuclear responses in VV-infected mouse lungs

Lung Compartments	Pathological Features	Severity grading ^c				
		Day 5			Day 7	
		Control + VV (n = 4) ^d	LCMV-immune + VV (n = 4)	MCMV-immune + VV (n = 2)	Flu-immune + VV (n = 2)	Control + VV (n = 11)
Alveoli	Edema AMI ^a	2.8 (2-3)	1.5 (0-3)	2.5 (2-3)	2	2 (0-3)
BALT	Extent of BALT		1.8 (1-3)	1	0.5 (0-1)	0.5 (0-2)
Airways	Necrotizing bronchiolitis	1.8 (0-3)		1	2 (1-3)	1.4 (0-3)
	Bronchiolitis	0.8 (0-2)	0.3 (0-1)	1	1	1.5 (0-3)
	Peribronchial AMI	2 (1-3)	0.5 (0-1)	1		1.2 (0-3)
	Peribronchial CMI			0.5 (0-1)		
	Bronchiolitis obliterans					0.6 (0-2)
	Bronchiolization				0.5 (0-1)	
Interstitialium	Consolidation CMI ^b		1.8 (1-2)	1	0.5 (0-1)	
	AMI	1.5 (0-3)		1.5 (1-2)	0.5 (0-1)	0.5 (0-3)
Pleura	CMI	0.8 (0-1)	1		1.5 (1-2)	0.2 (0-1)
						1

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Lung Compartments	Pathological Features	Severity grading ^c				
		Day 5			Day 7	
		Control + VV (n = 4) ^d	LCMV-immune + VV (n = 4)	MCMV-immune + VV (n = 2)	Flu-immune + VV (n = 2)	Control + VV (n = 11)
Vasculature	Lymphocytic cuffing and tufting		1 (0 - 2)	0.5 (0 - 1)	1.5 (0 - 1)	LCMV-immune + VV (n = 9)
	Necrotizing vasculitis					0.7 (0 - 2)
	Endothelial activation			0.5 (0 - 1)		0.3 (0 - 2)
	Perivascular CMI		0.5 (0 - 1)	3		0.4 (0 - 1)
	Perivascular AMI	2 (0 - 3)		1.5 (0 - 3)	1 (0 - 2)	0.8 (0 - 3)
	Edema	0.3 (0 - 1)				0.6 (0 - 3)

Five or seven days after VV infection the mouse lungs were harvested, sectioned and stained (H+E), as described in Materials and Methods. a, AMI, acute mixed infiltrate (polymorphonuclear and mononuclear cells); b, CMI, chronic mononuclear infiltrate (lymphocytes and macrophages); c, Grading of the pathological changes in the lung: based on the distribution and the severity of disease from 0 to 3 (blank or 0, within normal limit; 1, 1-9%, 2, 10-49%, and 3, ≥50% involvement of the lung parenchyma). d, the number of mice in each group. e, Data are presented as average of scores from multiple mice per treatment, and the number in parentheses after each average score represents the range of scores from mice challenged with the virus indicated.

7 of VV infection LCMV-immune mouse lungs also showed the less common pathology of bronchiolitis obliterans (Figure 29c, d and Table 19).

The effects of immunity to MCMV or influenza A virus on VV-induced immunopathology. Following VV challenge, the lungs of MCMV- and influenza A virus-immune mice, like LCMV-immune mice, showed a more prominent chronic mononuclear response (Figure 28k, l, 29e, g and Table 19), unlike the acute VV-infected controls that had acute neutrophilic infiltrates (Figure 29b and Table 19). Interesting, in both MCMV- and influenza A virus-immune mice there was a more dramatic accumulation of mononuclear cells around the vessels with less dramatic increases in BALF, while the reverse was true in LCMV-immune mice (Figure 28j, k, l, 29c, e, g and Table 19). Although alveolar edema was diminished in MCMV- and influenza A virus-immune mice, this was less so than in LCMV-immune mice (Table 19). The major differences between influenza A virus-immune mice and MCMV-immune mice upon VV challenge was the presence of mild residual areas of consolidation in VV-infected influenza A virus-immune mouse lungs, whereas VV-infected MCMV-immune mouse lungs demonstrated greater endothelial activation (Table 19). Clearly, prior immunity to any one of these three viruses led to dramatic alterations in VV-induced pathology in the lung. Although the common feature in this altered pathology was enhanced chronic mononuclear infiltrates, immunity to each virus resulted in a different pattern of pathology in the various compartments of the lung.

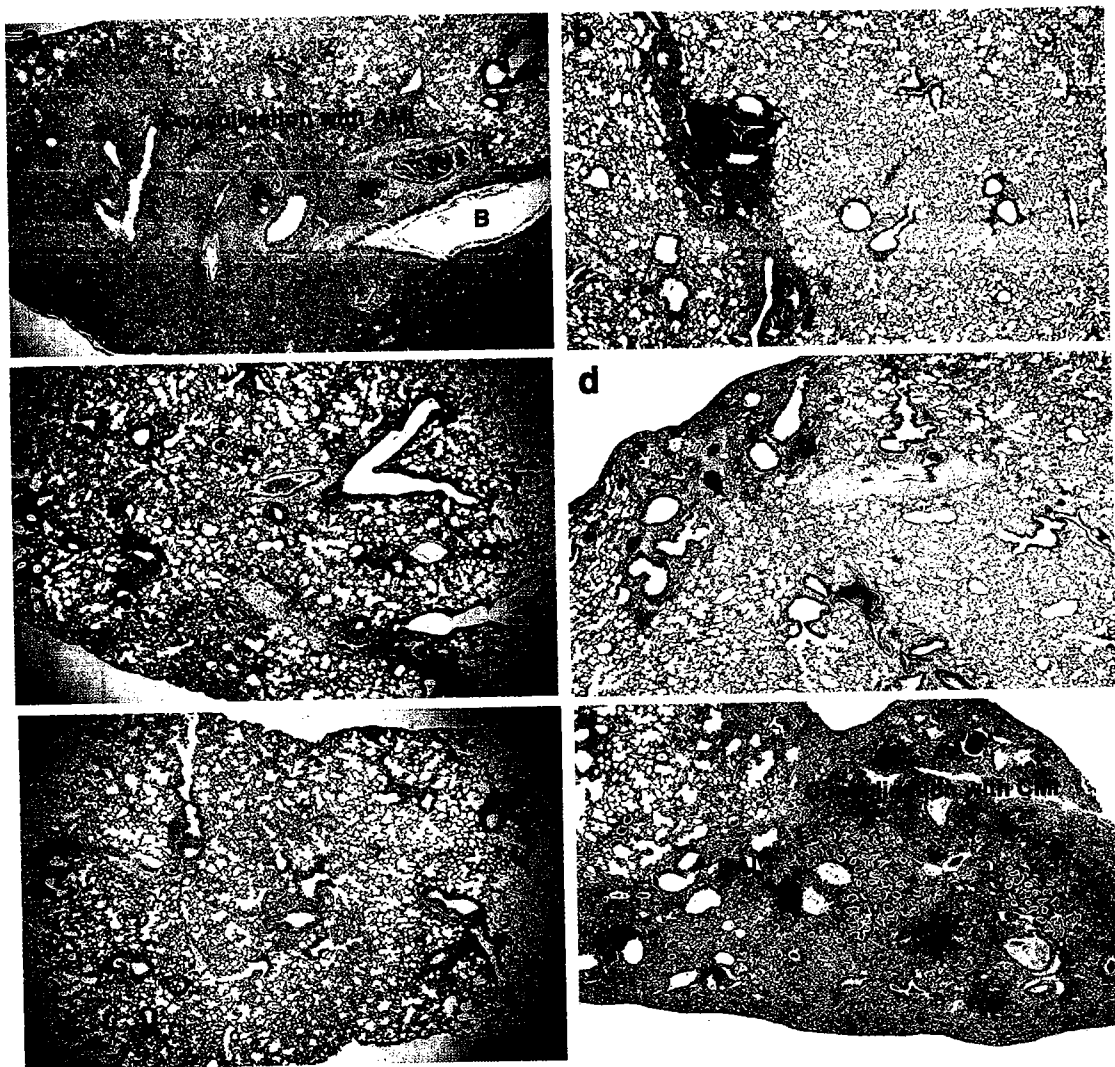


Figure 30. Prior immunity to Flu markedly enhances the usual mild mononuclear infiltrates in the lung upon LCMV- and MCMV-infection. Lung sections from mice were stained with H+E. Acute Flu infection (day 7) caused consolidation with AMI, composed of PMN and MN infiltrates (a), and Flu-immune lungs were essential normal with residual consolidated areas (b). As compared with a LCMV-infected lung (day 7) with mild lymphocytic response around airways and in the interstitium (c), LCMV-infected Flu-immune mouse lung interstitium showed slightly enhanced lymphocytic response and pneumonic consolidation composed of MN cells (d, day 7). Acute MCMV infection (day 7) induced interstitial disease with MN infiltrates and vascular lymphocytic tufting (e). Very strikingly, MCMV-infected Flu-immune lungs had increase in BALF and pneumonic consolidation of the interstitium (f). B: Bronchiole; V: vessel. Magnification: 4x of the objective of the digital light-microscope.

Table 20. Prior immunity to Flu enhances the usual mild chronic mononuclear responses in the lung upon LCMV and MCMV infection

Lung Compartments	Pathological Features	Severity grading ^c				
		Flu-Acute (n = 3) ^d	Flu-immune (n = 5)	Control + MCMV (n = 6)	Flu-immune + MCMV (n = 6)	Control + LCMV (n = 4)
Alveoli	Edema AMI ^a	1.3 (1 - 2)			0.2 (0 - 1)	0.5 (0 - 1)
		3				0.4 (0 - 1)
BALT	Extent of BALT	0.6 (0 - 1)	0.6 (0 - 2)	0.8 (0 - 1)	2.6 (1 - 3)	0
Airways	Necrotizing bronchiolitis	1 (0 - 2)				
	Bronchiolitis	1.6 (0 - 3)		0.2 (0 - 1)	0.2 (0 - 1)	
	Peribronchial AMI					0.3 (0 - 1)
	Peribronchial CMI			0.2 (0 - 1)		
	Bronchiolization		0.4 (0 - 1)		1.5 (1 - 2)	0
Interstitialium	Consolidation	2 (0 - 3)	0.6 (0 - 1)		2.5 (1 - 3)	0
	CMI ^b	3	1.8 (1 - 3)	2.2 (2 - 3)	2.8 (2 - 3)	1.3 (1 - 2)
	AMI	2.6 (2 - 3)				1.8 (1 - 2)
Pleura	CMI	2.3 (1 - 3)	1.2 (1 - 2)	2.2 (2 - 3)	2.8 (2 - 3)	1.3 (1 - 2)
	AMI	2.3 (1 - 3)				1.3 (1 - 2)

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Lung Compartments	Pathological Features	Severity grading ^c				
		Flu-Acute (n = 3) ^d	Flu-immune (n = 5)	Control + MCMV (n = 6)	Flu-immune + MCMV (n = 6)	Control + LCMV (n = 4)
Vasculature	Lymphocytic cuffing and tufting	1		2.2 (2 - 3)	0.8 (0 - 2)	0.3 (0 - 1)
	Necrotizing vasculitis	0.3 (0 - 1)				
	Perivascular CMI		0.4 (0 - 1)			0.3 (0 - 1)
	Perivascular AMI	3		1.3 (0 - 2)	0.6 (0 - 2)	0.8 (0 - 1)
	Endothelia activation					
	Edema					1 (0 - 3)

Seven days after infection the mouse lungs were harvested, sectioned and stained (H+E), as described in Materials and Methods. a, AMI, acute mixed infiltrate (polymorphonuclear and mononuclear cells); b, CMI, chronic mononuclear infiltrate (lymphocytes and macrophages); d, Grading of the pathological changes in the lung: based on the distribution and the severity of disease from 0 to 3 (*blank* or 0, within normal limit; 1, 1-9%, 2, 10-49%, and 3, ≥50% involvement of the lung parenchyma). d, the number of mice in each group. e, Data are presented as average of scores from multiple mice per treatment, and the number in parentheses after each average score represents the range of scores from mice challenged with the virus indicated.

Figure 31

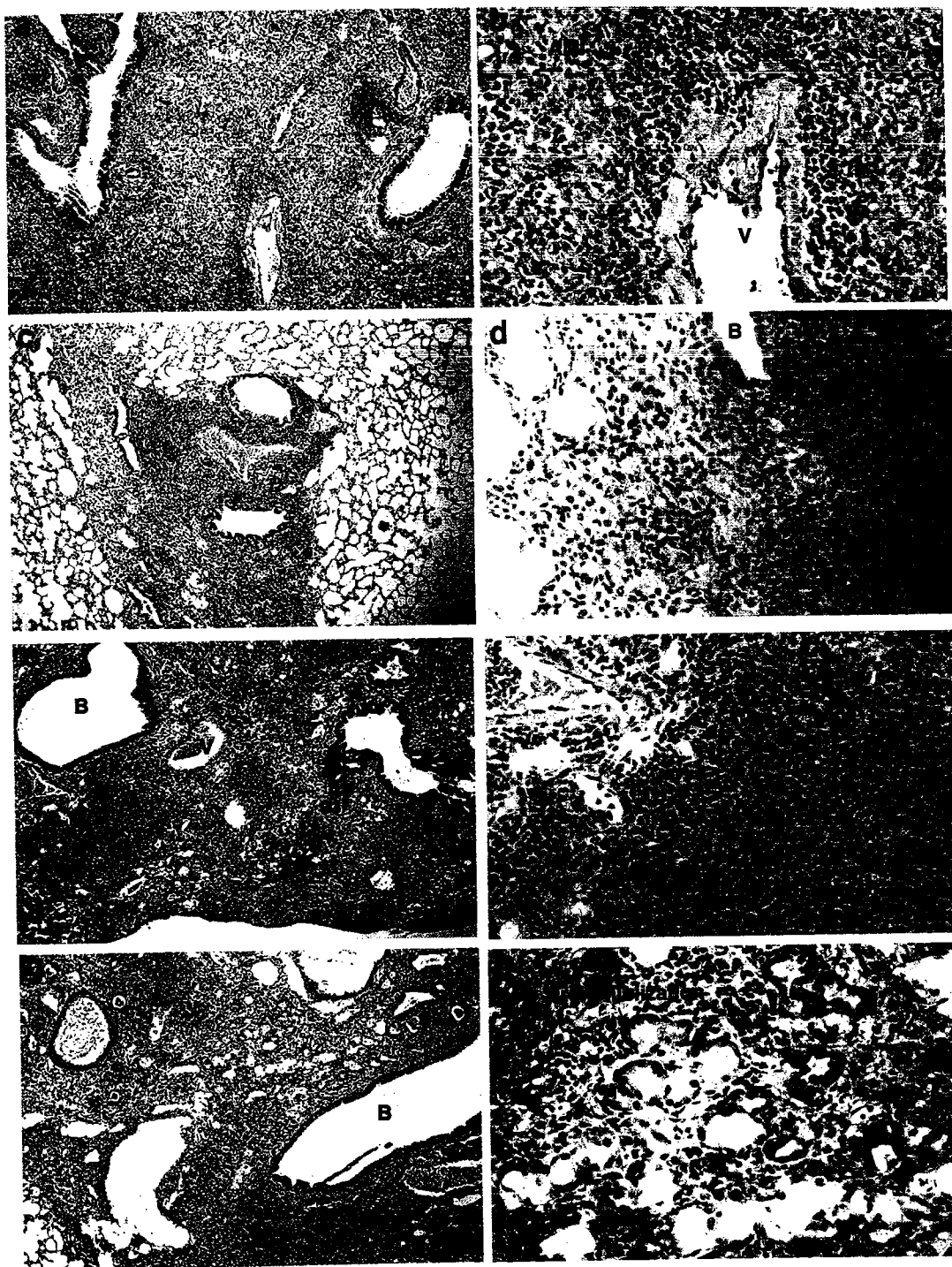


Figure 31. Unique immunopathology in MCMV-infected mouse lungs induced by prior immunity to Flu. Lung sections shown here were the same as the ones in panel a, b, and f of Figure 30 but were at higher magnifications. (a and b): an Flu-infected lung section at 10x and 40x magnification respectively 7 days after challenge. (c and d) a Flu-immune mouse lung section. (e and f) a MCMV-infected Flu-immune mouse lung section, showing very prominent chronic MN inflammation in a large densely consolidated area. (g and h) another MCMV-infected Flu-immune mouse lung section, showing an enhancement of alveolar bronchiolization.

F. Prior immunity to influenza markedly enhances the usual mild chronic mononuclear responses in the lung upon acute LCMV or MCMV infection

I was particularly interested in the influence that immunity to influenza A virus had on lung pathology upon challenge with MCMV or LCMV, as prior immunity to influenza A virus appeared to have a detrimental effect on clearance of these viruses. The most prominent feature of **acute influenza A virus infection** is severe pneumonic consolidation of the lung, which is associated with acute neutrophilic infiltration (Figure 30a, 31a, b and Table 20). When this resolves, the mouse is left with some minimal residual areas of consolidation (Figure 30b, 31c, d, and Table 20), which appear to be present for the lifetime of the animals as they were observed even 24 months after infection (not shown).

The effects of immunity to influenza A virus on MCMV-induced immunopathology. When influenza A virus-immune mice were infected with MCMV, the lung pathology was very different than VV infection of influenza A virus-immune mice but also very different than acute MCMV infection in control mice. Acute MCMV infection resulted in no pneumonic consolidation, but did induce a chronic mononuclear infiltration around bronchioles and vessels and in the interstitium (Figure 30e). Upon MCMV infection of influenza A virus-immune mice, the MCMV-induced lymphocytic response seen in the lung of non-immune mice was even further enhanced, with a dramatic increase in both BALF and a very striking pneumonic consolidation of the interstitium, but this consolidation was composed of chronic mononuclear infiltration rather than the acute neutrophilic infiltration observed during acute influenza A virus infection (Figure 30f, 31e, f and Table 20). There was also an enhancement of alveolar

bronchiolization, which did occasionally appear in a very mild form in influenza A virus-immune mouse lungs (Figure 30f, 31c, g, h and Table 20). Thus, prior immunity to influenza led to an overwhelming chronic mononuclear response, which was associated with decreased ability to clear MCMV (Table 16).

The effects of immunity to influenza A virus on *LCMV*-induced immunopathology. Interestingly, prior immunity to influenza A virus had a similar effect on the immunopathology in response to another virus, LCMV. Like MCMV, acute infection with LCMV in control mice results in predominantly a mild mononuclear infiltrate in the interstitium of the lung (Figure 30c and Table 20). However, in influenza A virus-immune mice LCMV infection induced an increase in chronic mononuclear infiltration around airways and vessels, increased bronchiolization, and some enhancement of pneumonic consolidation composed of chronic mononuclear cells (Figure 30d and Table 20). Prior immunity to influenza A virus was also associated with decreased ability to clear LCMV (Table 16). Thus, prior immunity to influenza A virus resulted in marked enhancement of the usually mild mononuclear infiltration that occurs upon LCMV or MCMV infection.

G. Brief summary and discussion

I have shown in this chapter that mice with respiratory infection with LCMV, MCMV or influenza A virus can clear the virus in the lung and enter a resting immune healthy state. When these immune mice are challenged intranasally with an unrelated virus, previous exposure to LCMV, MCMV and influenza A virus can result in protective immunity against VV, but prior immunity to influenza A virus can lead to enhanced

Table 21. Sequence of heterologous virus infections influences viral clearance, early cytokine profiles and histopathology in the lung

	Virus titer ¹	Cytokine profile ²	Histopathology ¹
LCMV-immune + VV vs. Acute VV	↓	IFN-γ ↑ IL-6 ↓	<ul style="list-style-type: none"> • Dramatically decreased edema • Increased chronic lymphocytic and MN responses • Decreased acute inflammatory (PMN) responses • Early increased prominent BALT (lymphocytic) • Bronchiolitis obliterans
MCMV-immune + VV vs. Acute VV	↓	ND ³	<ul style="list-style-type: none"> • Decreased edema • Increased chronic lymphocytic and MN responses, especially around vessels • Decreased acute inflammatory (PMN) responses • Moderate BALT • Endothelial activation
Flu-immune + VV vs. Acute VV	↓	IFN-γ ↑ IL-1β ↓	<ul style="list-style-type: none"> • Decreased edema • Increased chronic lymphocytic and MN responses • Decreased acute inflammatory (PMN) responses • Mild BALT • Mild consolidation
Flu-immune + LCMV vs. Acute LCMV	↑	ND	<ul style="list-style-type: none"> • Slightly increased BALT • Slightly increased MN response in the interstitium and around vessels • Moderate consolidation • Increased bronchiolization
Flu-immune + MCMV vs. Acute MCMV	↑	IFN-γ ↑ IL-6 ↑ IL-1β ↑ IL-12 ↑	<ul style="list-style-type: none"> • Increased prominent BALT (in the consolidated areas) • Increased consolidation with massive lymphocytic and MN infiltrates • Increased bronchiolization

1. day 5-7 postinfection; 2. day 3 postinfection; 3. ND, not done.

replication of LCMV and MCMV. No enhanced virus replication was observed among tested heterologous viruses in previous systemic i.p. infection studies (Selin et al., 1998). Therefore, respiratory mucosal infections with heterologous viruses might be a good model system to study the profound effect, either beneficial or detrimental, of one history of a virus infection on immunity and immunopathology upon a subsequent challenge with an unrelated virus (Table 21).

I also show that, in situations where prior immunity to LCMV or influenza A virus results in protective immunity against VV infection, there were enhanced Th1 type responses with increased IFN- γ and suppression of some pro-inflammatory responses upon VV challenge. However, the presence of pro-inflammatory cytokines in MCMV-infected influenza A virus-immune mice is associated with enhanced MCMV replication.

I have shown in this chapter that an acute respiratory infection of C57BL/6 mice with LCMV, VV, MCMV or influenza A virus causes distinct immunopathological changes in the lung and that, in the resting immune state, virus-challenged mouse lungs return to essentially normal architecture. When the LCMV-, MCMV- or influenza A virus-immune mice are challenged with VV, the immunopathology varies dramatically not only from the VV-infected controls but also from each other, depending on the prior heterologous virus exposure. The enhanced accumulation of mononuclear cells appears to be associated with the ability to decrease VV titers. I also show that prior immunity to influenza leads to an overwhelming chronic mononuclear response and ultimately decreased ability to clear MCMV. Similar to MCMV infection, LCMV infection of influenza A virus-immune mice induces a more prominent chronic mononuclear response, associated with enhanced LCMV replication in the lung than LCMV infection of non-immune mice.

Taken together, the sequence of infections with different viruses determines the consequences of heterologous antiviral immunity and immunopathology in the lung (Table 21).

CHAPTER VI

DISCUSSION

This thesis examines the activation and role of virus-specific memory CD8 T cells in heterologous antiviral immunity and immunopathology in the lung. A heterologous virus-induced modulation of memory CD8 T cells specific for a previously encountered virus is explored here in terms of the phenotypic and functional activation status of memory CD8 T cells, the kinetics of accumulation of these cells in lymphoid (the MLN and spleen) and non-lymphoid (the lung) organs, as well as the memory T cell repertoire alteration upon the heterologous viral infection. The roles of cross-reactivity and cytokines in activation of the heterologous memory CD8 T cells are also pursued. Associated with this modulation of virus-specific memory CD8 T cells by a heterologous virus, mortality upon a high dose challenge, the viral load, early cytokine profiles and lung immunopathology are examined.

In this thesis, I have provided evidence that the activation and modulation of LCMV-specific memory CD8 T cells occur in an organ-dependent manner upon infection with VV and that this activation and modulation process is associated with IFN- γ -mediated heterologous antiviral immunity and immunopathology in the lung. I also provide data suggesting that cross-reactive T cell responses and initial recruitment by virus-induced cytokines play a role in modulation of LCMV-specific memory CD8 T cells upon VV infection. Finally, I have made observations implying that the sequence of infections with different viruses determines the consequences of heterologous immunity and immunopathology. This is evidenced by the findings showing beneficial enhanced

VV clearance in LCMV, MCMV, or influenza A virus-immune mice and detrimental increased LCMV and MCMV replication in influenza A virus-immune mice. Taken together, the data presented in this thesis provide a better understanding of the immunity and pathogenesis to a pathogen in the natural environment as the host is exposed to multiple different pathogens over a lifetime. Below is an in depth discussion of the major points made in this thesis.

A. The frequency and hierarchy of virus-specific memory CD8 T cells in lymphoid and non-lymphoid organs of mice with a respiratory viral infection

The results that showed a high frequency of LCMV-specific memory CD8 T cells in the resting immune mice after resolution of respiratory LCMV-infection are consistent with contemporary studies on influenza A virus infection (Hogan et al., 2001a; Marshall et al., 2001), VSV infection (Masopust et al., 2001), and RSV infection (Ostler et al., 2001). Using LDAs and intracellular IFN- γ assays, I have shown that a high frequency of LCMV-specific memory CD8 T cells is present in the lung of LCMV-immune mice with a very slow decline over 1 year after resolution of LCMV infection. The proportion of LCMV-specific CD8⁺ T cells in the MLNs of these same mice remained stable (around 2% of CD8 T cells), and in the spleen it was also relatively stable but higher than in the MLNs. There are several possibilities accounting for this pattern. First, lymphocytes and lymphoblasts from the lung vascular pool differ from peripheral blood or splenic lymphocytes in their migration pattern (Binns et al., 1992). Once the activated CD8⁺ T cells have been recruited to the lung, they may not have easily re-circulated (Harris et al., 2002), whereas between secondary lymphoid organs long-lived memory T cells can

re-circulate. Secondly, T cells which localize to non-lymphoid organs have an increased ability to survive, as a recent study with annexin V (a pro-apoptotic cell surface marker) and TUNEL staining (for apoptotic cells) has shown much less susceptibility to apoptosis in memory CD8 T cells in peripheral organs such as the lung than cells in secondary lymphoid organs such as spleen and MLN (Wang, 2002). Third, once CD8 T cells migrate into the lung, these cells are unable to divide (Harris et al., 2002). Recently published studies identified two distinct populations of memory CD8 T cells in the lung airway lumina following recovery from a respiratory Sendai virus infection (Cauley et al., 2002; Hogan et al., 2002). A large population (90%) of Ag-specific CD8⁺ T cells persists in a functional state for several weeks with minimal further division after acute infection, while only a smaller population (10%) of Ag-specific CD8⁺ T cells is maintained in the lung airways by homeostatic proliferation and migration to lung airways from the lymphoid organs after viral clearance. All these features for memory T cells in the lung may explain why the frequency of memory CD8 T cells in the lung is high but wanes gradually.

The long-term persistence of memory CD8 T-cell populations in lymphoid organs may be due to homeostatic turning over at a slow, yet steady, pace (Razvi et al., 1995; Sprent et al., 1999). Since memory cells remain in a higher activation state than naïve cells, these memory cells can respond to stimulatory cytokines such as IL-15 and IL-7 (Goldrath et al., 2002; Ku et al., 2000; Schluns et al., 2000; Tan et al., 2002). Meanwhile, TCRs of memory CD8 T cells may also be stimulated by endogenous self-ligands, low-affinity cross-reactive ligands, or in some cases possibly by persisting cognate viral antigens (not necessary in infectious forms) (Gray, 2002). Two new mouse

models in which TCRs can undergo conditional deletion have been developed (Labrecque et al., 2001; Polic et al., 2001). The first model allows the inducible ablation of the TCR on mature T cells *in vivo* by Cre recombinase-mediated TCR α gene deletion (Polic et al., 2001). Since the Cre recombinase is under the control of type-I IFN promoter, this model is not suitable for a viral infection system which induces IFN. The second model exploited the binary transgenic strategy in which the first transgene encodes a tetracycline (tet)-sensitive transactivator and the second transgene encodes OVA257-264-specific OT-1 α chain and is controlled by the transactivator. Tet inhibits the transactivator binding to and activating the second transgene (Labrecque et al., 2001). These mice can first be primed with an antigen. After the primed T cells enter the memory phase, TCRs on those memory T cells can undergo deletion with an inducing agent. Then, the memory T cells with no TCRs can be tracked to examine their survival and functions. This strategy will help further clarify the role of TCR on memory T cell maintenance.

Another interesting but unexpected observation was that the specificity of the CD8⁺ T cell response to acute LCMV infection between MLN and lung differed, while the spleen tended to have a similar repertoire to the lung. There may be several possibilities accounting for this. During the priming phase, there could be differential expression of viral proteins in different organs, resulting in different magnitudes of expansion of different epitope-specific CD8 T cells. Recently, the functional heterogeneity of dendritic cell subsets has also been recognized in the spleen and MLN (Reis e Sousa et al., 1999; Shortman et al., 1997), so it is possible that different DC subsets prime CD8 T cells with different specificity distinctively in the spleen and MLN. Moreover, different tissue

adhesion molecules could possibly lead to different thresholds of TCR signaling. Alternatively, in different organs some virus-induced epitope-specific T cell populations may respond to cross-reactive tissue-specific self-antigens. Of note, and for unknown reasons, a lower proportion of GP33-specific cells bound Annexin-V than NP396- and NP205-specific cells (Wang, 2002), and this difference was more dramatic for T cells in the MLN than in the lung. This suggests that GP-33 cells in the MLN survived better than the cells with other specificities. This might be one of reasons for the difference in repertoire between the lung and MLN. The definitive evidence that explains this difference in repertoire between the lung and MLN has not yet been found. Nonetheless, heterologous VV infection modified the LCMV-specific repertoire differently in both these two organs in such a manner that it further enhanced the difference between these two sites, as discussed below.

B. Dynamic modulation of memory CD8 T cell repertoire specific to one virus during a heterologous viral infection.

Memory T cells exist in a dynamic network, and re-infection with **homologous** viruses can activate these memory T cells and modulate the original repertoire. For example, a recent study on adoptively transferred LCMV-immune leukocytes showed that homologous LCMV re-infection caused more than seven divisions of T cells specific for each epitope, with dramatic increases in number and slight changes in LCMV-specific epitope hierarchy with a better NP396- than gp33-specific response in the spleen (Kim et al., 2002; Selin et al., 1999). Another example was an alteration of the repertoire of influenza A virus-specific memory CD8 T cells following secondary challenge, and a

H2D^b-restricted epitope PA224-233 prominent in the primary influenza A virus-specific CD8 T-cell response was found much less apparent (Belz et al., 2000).

Heterologous viruses can also modulate the original memory CD8 T cell pool specific for a previously encountered virus. Since the replication of a heterologous virus is not constrained by neutralizing antibody, if this heterologous virus can activate memory T cells that are specific for a previously encountered pathogen, the high antigen load might lead to profound T-cell activation. Indeed, in this thesis, I observed that, upon i.n. challenge with heterologous VV, (i) LCMV epitope-specific memory CD8 T cells were activated by VV early in infection and were producing IFN- γ *in vivo* and cytolytically active; (ii) there was a selective expansion and modulation of the original LCMV-specific memory CD8 T cell repertoire; and (iii) there was also a selective organ-dependent redistribution of these defined antigen-specific heterologous memory T cell populations. Of interest, at day 3 post VV challenge, there was no significant number of CD4 T cells producing IFN- γ *in vivo* in both LCMV-immune and control mouse lungs. Since CD4 T cells was required to mediate heterologous immunity and altered immunopathology (Selin et al., 1998), future studies should further examine the dynamics of modulation of LCMV-specific memory CD4 T cells at later time points after VV infection. CD8⁺ T cells from LCMV-immune mice were found to be cytolytically activated by infection with heterologous viruses (Selin et al., 1999; Selin et al., 1996; Yang et al., 1989). The recent study on adoptive transfer of splenocytes from LCMV-immune mice into Thy-congenic mice also showed that infections with the heterologous VV caused more than seven divisions and increases in number of T cells specific to some putatively cross-reactive (e.g. GP33 and NP205-specific CD8 T cells) but not other

epitopes and resulted in substantial changes in the hierarchy of the LCMV-specific T cells (Kim et al., 2002). In another system with heterologous virus infections, LCMV and PV encode cross-reactive CD8⁺ T-cell epitopes (LCMV NP205 and PV NP205), which are subdominant in each infection. However, if LCMV-immune mice are infected with PV, or if PV-immune mice are infected with LCMV, the T-cell responses to these NP205-specific peptides become dominant, involving more than 20% of the CD8 T cells. Meanwhile, CD8 T cell responses to major immunodominant epitopes are significantly decreased in both cases upon heterologous viral challenge (Brehm et al., 2002). This new epitope hierarchy remains in the resting double immune state (LCMV and PV-immune or PV and LCMV-immune).

Of note, in some experiments (Figure 10), the percentage of LCMV-specific memory CD8 T cells in the lung was lower at day 3 post VV infection than that prior to VV challenge. This might be due to bystander apoptosis of memory T cells early in VV infection (McNally et al., 2001). At this early time point, chemokine-induced nonspecific recruitment and cytokine or cross-reactivity-driven proliferation of LCMV-specific memory CD8 T cells in the lung might not offset the loss of the cells. Nonetheless, LCMV-specific memory CD8 T cells in the lung were activated producing IFN- γ *in vivo* at day 3 post VV infection. Actually, the accumulation of LCMV-specific memory CD8 T cells in the lung was found to occur relatively slowly upon VV challenge, as it took approximately 12 days. Our laboratory previously showed that after acute LCMV infection of IFN- γ R KO mice the CD8 T cell response returned to homeostasis, but with delayed kinetics (Lohman and Welsh, 1998). This suggests that IFN- γ influences the contraction phase after the peak of CD8 T cell response to LCMV. One recent report

further showed a delayed contraction of secondary effector CD8⁺ T cells as compared to the contraction of primary effector CD8 T cells (Badovinac et al., 2002). The authors hypothesized that impaired IFN- γ signaling—whether by receptor modulation or induction of inhibitory molecules in secondary CD8⁺ effector T cells—could account for the delayed contraction of these populations. Is it possible that upon heterologous VV challenge, LCMV-specific memory CD8 T cells underwent a pseudo-secondary response with both a delayed expansion phase (due to a lower affinity interaction between VV antigens and LCMV-specific CD8 T cells as compared to homologous LCMV infection) and a delayed contraction phase, resulting in slow accumulation in the lung?

Modulation of memory CD8 T cells specific to one virus during heterologous viral infections is not a rare event. A study from another group showed that there was an increase in CTLp frequency, analyzed by LDA, of Sendai virus-specific memory CD8 T cells in the lung and spleen upon influenza A virus infection (Tripp et al., 1995a). Another study from the same group also showed that the relative prevalence of divided influenza A virus NP366-specific memory CD8 T cells (defined by BrdU⁺, which was used as a indicator for DNA synthesis) increased significantly in the mouse spleens 8 days after i.v. exposure to LCMV and that the prevalence of BrdU^{hi} LCMV NP396-specific memory CD8 T cells also increased significantly in the spleens 8 days after influenza A virus infection (Flynn et al., 1999). In a mouse model of RSV infection, if mice were infected with influenza A virus before the recombinant VV-RSV immunization and subsequent RSV challenge, there was an accumulation of influenza A virus NP366-specific CD8 T cells in the lung upon RSV challenge (Walzl et al., 2000).

C. The roles for T cell cross-reactivity and cytokine and chemokine milieu in activation and modulation of virus-specific memory CD8 T cells during heterologous viral infections.

There may be several factors driving or assisting activation and modulation of virus-specific memory CD8 T cells following the heterologous viral challenge. The first factor is the activation of memory CD8 T cells by a cross-reactive antigen. In this thesis, I provide several pieces of evidence suggesting that VV cross-reactive epitopes may play an important role in activation and modulation of LCMV-specific memory CD8 T cell populations. Upon heterologous VV challenge, there were preferential expansions of LCMV-specific memory CD8 T cells with discrete specificities (GP33- and /or NP205-specific). VV infection can thus markedly alter the LCMV-specific memory T cell hierarchy, suggestive of cross-reactive responses. Our previous study showed that PV and VV infections of LCMV-immune mice induced the reactivation of memory CTL specific to LCMV. LDAs demonstrated that at least part of this reactivation of memory cells in LCMV-immune mice related to cross-reactivity at the clonal level (Selin et al., 1994). Studies on adoptively transferred CFSE-labeled LCMV-immune donor T cells into Thy-1 congenic hosts inoculated with VV also found that VV often induced preferential expansions in NP205- and gp33-specific T cells (Kim et al., 2002). In another system, a well-characterized cross-reactivity between LCMV NP205 and PV NP205 dramatically influenced the immunodominance in LCMV- or PV-immune mice upon PV or LCMV heterologous challenge (Brehm et al., 2002). In this thesis, I have shown that VV p1, a peptide partially homologous to LCMV NP 205, stimulated LCMV-NP205 specific CD8 T cells, suggesting that NP205 may be the cross-reactive epitope. However, the

percentage of NP205-specific CD8 T cells that recognized VV p1 did not increase 12 days after VV challenge, suggesting the unknown cross-reactive VV epitopes other than VV p1 might be responsible for activation and selective expansion of NP205 T cells. Moreover, because of the caveat of the technique used (double stains of cells with anti-IFN- γ mAb for VV-peptide-induced intracellular IFN- γ and NP205 dimer/tetramer for NP205-specific TCRs), the results could not negate the possibility that VVp10 and p24, the two other peptides with partially homology to LCMV NP 205, also cross-reactivate LCMV NP205-specific memory CD8 T cells. Furthermore, since VV encodes about 200 proteins, it is highly possible that there are more VV epitopes cross-reactive with other LCMV epitope-specific CD8 T cells. In some experiments (Figure 6), a more dramatic increase in NP205-specific CD8 T cells was observed in the lung than in the MLN 12 days after VV challenge. This might be due to the fact that VV still presented in the lung while VV was cleared in the MLN by day 12 postinfection. The cells recruited to the lung might undergo further proliferation upon continuously encountering their cross-reactive antigens.

Future studies should use new strategies to identify cross-reactive epitopes. For instance, cross-reactive epitopes can be defined by assessment of epitope-specific dimer or tetramer-sorted fresh CD8 T cells for their ability to respond to potentially cross-reactive antigens. The sorted cells will be used only after a defined rest period allowing bound dimers or tetramers to fall off the cell surface, because their presence on the cell surface may block the interaction of that cell with the potentially cross-reactive antigens. If the frequency of the cross-reactive T cells is so low, then the sorted cells will need to be plated into LDAs to obtain a cross-reactive frequency. Then defined peptide-specific

cross-reactive CD8 T cell clones will be generated to assess the effect of these clones in generating immunity to heterologous viruses. Alternatively, the role of cross-reactivity in heterologous immunity can be tested by examining the effect of immunization with cross-reactive peptides or minigenes that encode the cross-reactive epitopes (Rodriguez et al., 1997) on generating immunity to heterologous viruses.

Several issues should be considered in terms of cross-reactivity. **First**, a cross-reaction that involves the same determinants on the TCR might be easier to predict by searching for similar amino-acid side chains at positions of peptides that engage the TCR. This method has been used for the calculation of potential frequencies of cross-reactivity (Mason, 1998). **Second**, if different determinants on the TCR cross-react with different peptide-MHC structures, it would be very difficult to predict when such cross-reactivity would occur. A way to overcome the difficulty in defining this type of cross-reactivity may be a peptide-induced intracellular cytokine assay, which has an advantage of sorting out the cross-reactive epitope since a large pool of peptides can be tested within the same assay. The **third** issue about cross-reactivity is that a given T cell may express two different TCRs. This could happen due to incomplete allelic exclusion of the second TCR α -chain (Alam and Gascoigne, 1998). Although the extrapolated frequency of these cells present in the normal repertoire ranges from 7-21% (with an average of 15%), the frequency of antigen-responsive cells expressing two surface α chains is relatively low (<1%) (Heath et al., 1995). This raises the possibility that dual α chain T cells may have a selective disadvantage in responding to specific antigen. A simple way to test the role of dual receptor T cells in heterologous immunity would be to make a F1 hybrid of a TCR α KO (B6 background) and B6 mice. This mouse would only express one

a-chain. A study on CD8 T cells from PV-infected LCMV-immune F1 offspring of a TCR α KO B6 and normal B6 mice still showed a high degree of cross-reactivity between LCMV NP205 and PV NP205 (Brehm et al, unpublished), suggesting that cross-reactivity is not due to dual TCRs. The **fourth** issue about cross-reactivity is that possibly only a subpopulation of the T cells that are specific for a peptide will recognize the cross-reactive peptide, making T cell cross-reactivity more difficult to define. Because the TCR usage differs from host to host and stochastic elements might determine clonal dominance (Lin et al., 2000; Lin and Welsh, 1998), the proportion of peptide-specific T cells that cross-react with another peptide might differ from one host to another. This may be the reason why there was some variability in the alteration of the LCMV-specific memory CD8 T cell repertoire upon VV challenge between the experiments I have done. In the lung there was always a marked change in the selective specificities, with either the GP33- or NP205-specific responses dominating the repertoire. Adoptive transfer of T cells from a single LCMV-immune host into naïve donors should make the repertoire alteration less variable between different recipient mice upon VV challenge. By this method, potential cross-reactive epitopes between these two viruses will be defined more easily than studying the cells pooled from individual mice.

The second factor that may assist activation and modulation of memory CD8 T cells during the heterologous viral infection is the non-specific activation by cytokines — and potentially chemokines— induced by the virus. The cytokine milieu induced by any particular virus can be altered by prior exposure to other pathogens. In this thesis, I have shown that the respiratory infection of LCMV-immune mice with VV stimulates in the lung a significantly higher production of the T_H1-type cytokine IFN- γ but a lower

production of the pro-inflammatory cytokine IL-6 at days 3 postinfection than did non-immune controls. Moreover, the respiratory infection of influenza A virus-immune mice with MCMV results in greatly enhanced T_H1 type and proinflammatory cytokines. These altered cytokine profiles may have a impact on heterologous memory CD8 T cells, since memory CD8 T cells express or can rapidly up-regulate receptors for those cytokines.

Poly I:C, presumably through induced cytokines, can have a limited transient effect on memory CD8 T cells. I have provided evidence that both poly I:C and VV infection could lead to up-regulation of CD122 (IL-15R β and IL-2R β) on CD8 T cells in LCMV-immune mouse lungs, enabling these CD8 T cells to respond to IL-15 or IL-2 induced in those mice. Currently, it is unclear whether CD122⁺ CD8 T cells are true antigen-specific memory cells or memory-phenotype (CD44^{hi}) cells. There is one recent study showing that in the primed TCR transgenic mice at least two subsets of memory phenotype CD8 T cells coexist: CD44^{int}CD122⁻ CD8 cells, which are generated only following antigenic peptide priming; and CD44^{high}CD122⁺ CD8 T cells, which are heterogeneous in terms of antigenic specificity but can contain a fraction of Ag-specific memory cells (Walzer et al., 2002). A recent study showed that IL-15 was required for bystander proliferation induced by IFN, basal homeostatic proliferation, and survival of CD44^{high} CD122⁺ CD8⁺ cells (Judge et al., 2002). Poly I:C treatment of LCMV-immune mice resulted in a transient increase but no repertoire alteration of LCMV-epitope-specific CD8 T cells. These cells did not produce IFN- γ *in vivo*. Examining adoptively transferred CFSE-labeled LCMV-immune donor T cells into Thy-1 congenic hosts found that poly I:C stimulation caused a limited synchronized division of memory CD8 T cells specific to each of five LCMV epitopes, with no increase and sometimes a loss in

number, and no change in their epitope hierarchy (Kim et al., 2002). Of note, stimulation of mice with the type-I-IFN inducer poly I:C induces first apoptosis and a substantial loss (> 50%) of memory CD8⁺ T cells. Then, it seems that IL-15 stimulates the division of the remaining CD8⁺ T cells, such that they restore the CD8⁺ T-cell pool (McNally et al., 2001). Since poly I:C is a potent inducer of type I IFN and IL-15, type I IFN and IL-15 might play a partial role in increasing LCMV-specific CD8 T cells upon VV challenge. In the case of heterologous VV infection, LCMV-specific memory CD8 T cells may be induced into cell cycle by IL-15. This is a homeostatic process (Zhang et al., 1998) that is necessary because of attrition of memory CD8 T cells during the early virus-induced T cell and IFN responses (McNally et al., 2001).

However, it is difficult to exclude a role for TCR triggering in this process. I have shown that, after poly I:C treatment, there was a slight increase in CD69 expression on the total CD8 T cell population in the LCMV-immune mouse lung. Rapid transient up-regulation of CD69 has been linked to engagement of the TCR on T cells (Testi et al., 1994). There is one report showing that proliferation of CD44^{hi} CD8⁺ T cells followed by injection of poly I:C was not associated with significant up-regulation of CD69 or CD25 expression, which implies that TCR signaling was not involved (Tough et al., 1996). However, the type I IFN induced by immunostimulatory CpG DNA/ODNs (a mimic of microbial DNA and used as an adjuvant for immunization) can induce partial activation of naive T cells, leading to up-regulation of CD69 and other molecules but no entry into cell cycle (Sun et al., 2000). The receptors for dsRNA (poly I:C) and CpG DNA/ODNs, Toll-like receptor (TLR)-3 and TLR-9, respectively, on APCs have been found (Alexopoulou et al., 2001; Kaisho et al., 2001). It is possible that poly I:C itself or

cytokines induced by poly I:C might activate APCs, whose upregulated MHC-self peptide complexes could trigger TCRs on T cells, leading to CD69 expression, because all TCRs have some low level of reactivity against endogenous MHC-self peptide complexes. Therefore, it is difficult to exclude a role for TCR triggering following poly I:C treatment. Nonetheless, cytokines induced by poly I:C alone could only cause transient effects on CD8 T cell responses without proper antigenic stimulation.

IL-12 may play a role in modulation of memory CD8 T cells following the heterologous viral challenge. First, I observed that there was a slight increase in the frequency of LCMV-specific memory CD8 T cells in the IL-12KO LCMV-immune mice. This might be due to higher antigen load-driven stronger expansion of LCMV-specific CD8 T cells in IL-12KO mice than in normal B6 mice during acute LCMV infection. Moreover, since IL-12 enhances IFN- γ synthesis in activated T and NK cells (Trinchieri, 1995), and IFN- γ influences the contraction phase after the peak of CD8 T cell response to LCMV (Lohman and Welsh, 1998), the contraction of LCMV-specific CD8 T cells after the peak of the response was slightly impaired in IL-12KO mice, leading to a slight increase in the frequency of LCMV-specific memory CD8 T cells. I also observed that the GP33-specific memory CD8 T cell population was no longer predominant in the MLN of IL-12KO LCMV-immune mice. This observation may be explained by some reports stating that apoptosis of T cells via the Fas-FasL system can be suppressed by IL-12 (Fuss et al., 1999; Li et al., 2001; Marth et al., 1999; Neurath et al., 2001). The mechanism by which IL-12 counteracts the Fas pathway is not yet fully understood, although it is known that it does not involve the induction of the anti-apoptotic proteins, Bcl-2 or Bcl-xL, and thus may act on mitochondria-independent apoptosis signaling

events (Neurath et al., 2001). Moreover, a lower proportion of pro-apoptotic Annexin-V-binding GP33-specific cells was seen than of other specificities (e.g. NP396 and NP205), particularly in the MLN. This phenomenon might be associated with a Fas-FasL interaction (Wang, 2002). The above observations suggest that in the MLN of LCMV-immune IL-12KO mice, GP-33 specific cells and cells with other specificities undergo similar levels of apoptosis, given that the repertoire in the resting immune state reflects the hierarchy of LCMV-specific CD8 T cells at the peak of T cell response to acute LCMV infection. Therefore, GP33-specific cells no longer predominate in the MLN of LCMV-immune IL-12KO mice.

Secondly, upon VV infection, there was an increase in both CD69 and CD44 expression on the total CD8 T cell populations from LCMV-immune IL-12KO mice, suggesting activation of memory CD8 T cells in the absence of IL-12. However, the up-regulation of CD122 expression on CD8 T cells early in VV infection was impaired in the LCMV-immune IL-12KO mice. I postulate that IL-12 might play a role in up-regulation of CD122 expression on CD8 T cells early (day 3) after viral challenge. Since CD122 is shared by IL-15R and IL-2R, IL-15 secreted by APCs or IL-2 produced by memory CD4 cells early after viral challenge could modulate functions of CD8 T cells. Indeed, I found that VV challenge of LCMV-immune IL-12KO mice results in a slightly decreased accumulation of LCMV-specific CD8 T cells 7 days after VV challenge. Recently, IL-12 has been reported to drive memory CD8 T cells into homeostatic cell division *in vivo* (Kieper et al., 2001) and this effect is IFN- γ or IL-18-dependent and possibly mediated by IL-15 signaling (Tough et al., 2001). Bystander proliferation of memory CD8 T cells was also observed upon NKT or T cell activation by superantigens, and this process

could be induced by an IFN- α/β -independent, but IL-12- or IFN- γ -dependent pathway (Eberl et al., 2000). It is possible that the slightly decreased accumulation of LCMV-specific CD8 T cells in VV-challenge of LCMV-immune IL-12KO mice may be due to lack of IL-12-assisted proliferation of VV-activated LCMV-specific memory CD8 T cells. Since IL-12 is not a major growth factor for T cell proliferation (Trinchieri, 1995), the decrease of LCMV-specific CD8 T cells in VV-infected LCMV-immune IL-12KO mice was modest.

Thirdly, there was a dramatic reduction of *in vivo* IFN- γ production by LCMV-specific CD8 T cells in the lung of the VV-challenged IL-12KO mice. This suggests, as would be expected, that IL-12 is important to induce IFN- γ production in memory CD8 T cells. It is difficult to exclude a role for TCR triggering in this process, since I found no exogenous IL-12-induced enhancement of IFN- γ production by LCMV-specific CD8 T cells from LCMV-immune mice in the absence of any *in vitro* peptide stimulation in the 5-hour culture. There is one report showing that bystander activation of CD8 T cells by a combination of IL-12 and IL-18 (in a 48-hr *in vitro* culture of splenocytes) contributed to the rapid production of IFN- γ (Lertmemongkolchai et al., 2001). These authors concluded that this was TCR-independent, but even here, it is hard to rule out that TCR engagement by endogenous self-antigens occurs during the 2-day culture process. The definitive approach to test this hypothesis would be study those two mouse models in which TCRs can undergo conditional deletion (Labrecque et al., 2001; Polic et al., 2001). The memory cells from those primed mice can be treated with the inducing agents, and TCRs on those memory T cells will undergo conditional deletion. Then, the memory T cells with no TCRs can be examined for IFN- γ production in the presence of exogenous IL-12 and /or IL-18. Although there was a dramatic reduction of *in vivo* IFN- γ

production by LCMV-specific CD8 T cells in the lung of the VV-challenged IL-12KO mice, two preliminary experiments showed no decrease in the heterologous immunity against VV in LCMV-immune IL-12KO mice when compared to normal LCMV-immune mice upon VV infection. This result suggests that cytokines other than IL-12 may play a role in the heterologous immunity against VV during the respiratory VV infection.

Memory T cells express distinctive chemokine receptors and can migrate into areas of inflammation in a non-specific manner (Kunkel and Butcher, 2002). This non-specific migration might account for the increase in each LCMV epitope-specific CD8 T cell population early in VV infection. Moreover, some cytokines can influence cell migration by affecting expression of chemokines or chemokine receptors. There is one study showing a critical role for IL-12 in CCR5 (a chemokine receptor implicated in T cell migration to inflammatory sites) induction on TCR-triggered mouse CD8 T cells (Iwasaki et al., 2001). LCMV-specific CD8 T cells in VV-infected LCMV-immune IL-12KO mice may not migrate efficiently from the secondary lymphoid tissue such as the spleen into the inflamed lung, hence decrease in their number compared to normal mice. IFN- γ is a cytokine that can participate in inducing chemokines to recruit activated lymphocytes to the site of inflammation (Mahalingam et al., 2000). In my study, the early enhanced IFN- γ production may further recruit activated LCMV-specific memory CD8 T cells into areas of inflammation in a non-specific manner. This increases the probability that a memory T cell will encounter its up-regulated endogenous or cross-reactive ligands and become activated with the assistance of cytokines.

Three other cytokines might also potentially play a role in modulation of memory CD8 T cells during the heterologous viral infection. IL-18, originally called IFN- γ -inducing

factor, exhibits pleiotropic immunomodulatory activities. IL-18 possesses biochemical similarities to IL-1 and functional similarities to IL-12 (Dinarello, 1999). First, IL-18, in synergism with IL-12, has been recently reported to drive memory CD8 T cells into homeostatic cell division (Tough et al., 2001). Secondly, IL-18 mRNA and the precursor form of its protein are found in normal rat lung and they can be up-regulated in acutely inflamed lungs (Jordan et al., 2001). IL-18 was showed to be required for optimal IFN- γ release in the mouse lung during respiratory viral infection (Xing et al., 2000). Thirdly, one recent study showed that the IL-18-elicited anti-VV effect in the acute phase of infection of BALB/c mice was mediated by the sum of various host defense mechanisms including NK cells and CTL (Tanaka-Kataoka M, 1999), and another study showed selective IL-18 requirements for induction of local IFN- γ responses during MCMV infection (Pien et al., 2000). Therefore, IL-18 may be a good candidate for studying the role of cytokines in heterologous immunity in the future. In addition, a recently described IFN- γ inducing cytokine, IL-23, which is produced by monocytes /macrophages, acts primarily on memory T cells (Oppmann et al., 2000). Like IFN- γ , TNF- α is rapidly generated after T cells encounter viral peptides bound to the MHC (Slifka et al., 1999). The roles for IL-18, IL-23 and TNF- α in modulation of virus-specific memory CD8 T cells during heterologous viral infections should be investigated in future studies.

Taken together, the activation and modulation of memory T cells by heterologous agents is a very complex issue. It may be that some combination of cross-reactive memory T cells and the appropriate cytokine environment as well as

recruitment by chemokines results in activation and modulation of heterologous memory T cell populations.

D. Altered pathogenesis to a virus in association with virus-induced modulation of memory CD8 T cells specific for another unrelated virus

The modulations of memory CD8 T cells specific for a previously encountered virus may alter the pathogenesis of unrelated viral infections. These memory CD8 T cells activated by heterologous viruses may influence viral clearance, cytokine responses and virus-induced immunopathology. In this thesis, I have shown that memory CD8⁺ T cells specific to LCMV were activated *in vivo* by VV to produce IFN- γ in the lungs of LCMV-immune mice as early as 3 days after VV challenge. Then, there was a selective accumulation of these functionally activated LCMV epitope-specific CD8 T cells and a repertoire alteration in the lung. These modulations of LCMV-specific memory CD8 T cells were associated with protection against a lethal VV challenge and a decreased VV load throughout the course of the acute VV infection. In contrast with the VV-infected control mouse lungs that mainly showed severe edema and acute inflammatory infiltrates with PMN and MN cells, the VV-infected lungs of LCMV-immune mice had an altered immunopathology, characterized by prominent mononuclear infiltrates and the accumulation of LCMV-specific memory CD8⁺ T cells in the expanded BAL. This contributed to a marked change in the architecture of the lung during VV infection of LCMV-immune mice. I postulate that the accumulation of LCMV-specific memory CD8⁺ T cells in the lung may also lead to more rapid viral clearance, thus decreasing the acute edema and inflammatory infiltration. Since VV is sensitive to IFN- γ (Muller et al., 1994),

elevated levels of IFN- γ , which were detected in LCMV-immune mice and enhanced by IL-12, played an important role in mediating the protective heterologous anti-viral immunity and altered lung immunopathology. CD8 T cells were shown to be a source of IFN- γ , but involvement of CD4 T cells is also possible. The previous studies have shown that protection against VV in LCMV-immune mice depends on both CD8⁺ and CD4⁺ T cells in the systemic infection model (Selin et al., 1998). Even though NK cells made IFN- γ in response to VV infection, they did not contribute to the protective heterologous immunity against VV in LCMV-immune mice. The role of B cells in heterologous antiviral immunity should be examined in the future.

It was noted that the reduction of VV titer in the MLNs was more striking than the reduction of VV titer in the lungs of mice adoptively reconstituted with LCMV-immune splenocytes. There are some possibilities accounting for this result. First, LCMV-immune splenocytes may home better to lymphoid tissues like lymph nodes than to the peripheral tissues such as the lung. Generally, via i.v. adoptive transfer, cells isolated from lymphoid organs home better to lymphoid organs than to the peripheral tissues (Reinhardt et al., 2001; Rodolfo et al., 1990). A different approach using intratracheal adoptive transfers of bronchoalveolar lavage (BAL) cells isolated from the Sendai virus-immune mice into naïve mice observed a significant protection against a challenge with Sendai virus (Hogan et al., 2001b). The intratracheal route of cell transfer worked in that system because Sendai virus infection induces potent lymphocytic infiltrates in bronchiolar and alveolar spaces, and thus transferred BAL cells could home efficiently into the lung via the trachea. However, my research system is different from this study in that LCMV infection induces mainly mononuclear interstitial infiltrates, and

lymphocytes are seldom seen in airways (Figure 28b). Thus, intratracheal transfer of LCMV-immune cells isolated from the lung might not provide better protection than i.v. adoptive transfer did. Secondly, VV is cleared more rapidly in lymphoid tissues (Table 2). Thirdly, it is easy for a virus to infect permissive bronchiolar and alveolar epithelia cells and spread infectious viral particles through open air spaces within a short incubation period (Wright, 1997). During an i.p. infection with 10^6 PFU VV, VV is cleared in the spleen 5 days after infection and in peripheral tissues (fat pads and liver) by day 7 postinfection (Selin et al., 2001). In contrast, since VV replicated very well in the lung, the VV dose used for i.n. inoculations had to be decreased about 100-fold. The differences in VV titers between the immune group and the control group following i.n. challenges were also smaller than the ones observed in previous studies with systemic i.p. infections (Selin et al., 1998). Since mice are very sensitive to respiratory VV infection and VV replicates very well in the lung, the limited numbers of splenic LCMV-specific CD8 T cells homing to the lung after i.v. adoptive transfer of LCMV-immune splenocytes may not control VV efficiently. Nonetheless, adoptive transfer studies showed that the protective heterologous immunity against acute respiratory infection with VV was dependent on LCMV-immune splenocytes.

Two features of altered immunopathology observed in the lung of VV-infected LCMV-immune mice have significant implications for studying immune responses and immune-mediated diseases in the human lung. One was the dramatic expansion of BALT, implying that an individual's past history of infections may influence whether there would be an induction of BALT in response to an additional antigenic challenge. In human and mouse lungs BALT is not a constitutive structure, but BALT can be initiated

and vigorously expanded upon infection to become a highly integrated mucosal immune system along the respiratory tract (Pabst, 1992; Tschernig and Pabst, 2000). Another important feature observed in VV-infected LCMV-immune mouse lung was the development of bronchiolitis obliterans — an obstruction of the bronchiole by plugs of fibrin and inflammatory cells (Schlesinger et al., 1998) — in some mice. In humans, the etiology of this condition is not well understood, and it is thought to be immune-mediated and to occur in association with viral and intracellular bacterial infections or in response to some drugs (Schlesinger et al., 1998). Bronchiolitis obliterans also remains the most significant cause of morbidity and mortality following lung transplantation (McKane et al., 2001). Acute rejection is the main risk factor for the development of bronchiolitis obliterans and is characterized by a perivascular/bronchiolar leukocyte infiltration. One recent study showed that recruitment of CXCR3-expressing mononuclear cells plays an important role in the recruitment of mononuclear cells (Belperio et al., 2002). Our mouse model suggests that an individual's past history of infections may influence whether they develop bronchiolitis obliterans in response to an additional antigenic challenge. Since there are few mouse models of bronchiolitis obliterans available, studying this type of model might provide further insights into this poorly understood condition.

E. The sequence of infections with different viruses determines the consequences of antiviral immunity and immunopathology in the lung

The study presented in this thesis also examined what effect, if any, prior immunity to a virus other than LCMV had on immunity and immunopathology in

response to a heterologous virus challenge. The study clearly demonstrates that the specific sequence of heterologous virus infections can alter the course of subsequent virus infections. First, mice were immunized to three different viruses, LCMV, MCMV or influenza and I examined the impact immunity to each of these viruses had on the outcome of acute VV infection. Second, the mice were immunized with one virus, i.e. influenza A virus, and I examined the impact of immunity to influenza A virus on the acute responses to three different viruses LCMV, MCMV and VV.

These studies clearly indicate that each individual response to acute VV infection can be strongly influenced by that individual's past history of infections. There were many common features in the manner in which each of these viruses influenced the acute VV response, but immunity to each different virus also brought unique changes. Interestingly, immunity to any of the three viruses, LCMV, MCMV or influenza A virus resulted in significantly decreased VV titers in the lung compared to a non-immune host. In LCMV and influenza A virus-immune mice this protective immunity to VV was associated with enhanced T_H1 type responses, with increased $IFN-\gamma$, and suppressed pro-inflammatory responses, with decreased IL-6 or IL-1 β . A preliminary experiment also showed that there was no increase in T_H2 type cytokines (IL-4 and IL-5) in LCMV-immune mouse lungs upon VV infection. The fact that immunity to any of these three viruses was protective is most likely due to the fact that VV is very sensitive to $IFN-\gamma$ (Muller et al., 1994). Upon examining the pathology there was a decrease in alveolar edema in all three groups of mice (Table 21). However, in the VV-infected MCMV- and influenza A virus-immune mice there was a less effect on edema than in LCMV-immune mice. Prior immunity to any one of these three viruses did not result in the usual VV-

induced acute inflammatory response in the lung, but rather a predominantly mononuclear infiltrate (Table 21). However, the pattern of the lymphocytic infiltrates dramatically differed between the groups. In the LCMV-immune mice there was a pronounced lymphocytic accumulation in the BALT and around the airways while in MCMV and influenza A virus-immune mice there was more lymphocytic accumulations around the vessels. The major difference between influenza A virus-immune and MCMV-immune mice upon VV challenge was the presence of mild residual areas of consolidation in VV-infected influenza A virus-immune mouse lungs and the endothelial activation in VV-infected MCMV-immune mouse lungs. Therefore, immunity to LCMV, MCMV and influenza A virus resulted in enhanced chronic mononuclear infiltrates in the VV-infected mouse lungs; however, depending on prior immunity to different viruses, these were distinct patterns of pathology in those mouse lungs as summarized in Table 21.

Immunity to influenza A virus had a significant impact on the outcome of subsequent acute infections with any of three viruses, VV, LCMV and MCMV. Although immunity to influenza A virus resulted in some common features in the subsequent infection such as enhanced mononuclear infiltrates in the lung and increased early IFN- γ production, immunity to influenza A virus had a very different impact on subsequent infections, depending on the specific virus infection. As noted above, upon heterologous VV challenge, there was the beneficial effect of enhanced VV clearance in the lung. This was associated with an enhanced T_H1 type cytokine response with increased IFN- γ and suppressed pro-inflammatory cytokine responses when compared to the non-immune control mice. In marked contrast, influenza A virus-immune mice demonstrated

decreased ability to clear either MCMV or LCMV. Interestingly, the influenza A virus-immune mice infected with MCMV, had enhanced both T_H1 type cytokines such as, $IFN-\gamma$ and pro-inflammatory cytokines such as $IL-1\beta$, $IL-12$ and $IL-6$. This contrasts with the cytokine profile induced in VV-infected influenza A virus-immune mice. Upon LCMV or MCMV infection of influenza A virus-immune mice there was a dramatic enhancement of the mononuclear infiltration of the lung, which normally occurred in mild form in non-immune lungs (Table 21). MCMV infection pathology differed from LCMV induced pathology in that there was an increase in consolidation observed in MCMV-infected influenza A virus-immune lung. This consolidation was composed of dramatically increased chronic mononuclear infiltrations. It is interesting to note that when prior immunity to influenza A virus further enhances what would normally be a mild lymphocytic response in a MCMV- or LCMV-infected non-immune host, this enhancement of the lymphocytic response has a detrimental impact on MCMV or LCMV clearance (Table 21). However, when prior immunity transforms an acute inflammatory response to a predominant lymphocytic response as in influenza A virus-, LCMV- or MCMV-immune mice challenged with VV, there is an enhanced clearance of VV. Thus, it is clear that the specific sequence of virus infections controls the outcome of disease and depending on the infection sequence, exposure to heterologous viruses can be either beneficial, resulting in protective immunity or detrimental, leading to enhanced virus replication.

Two features of the altered immunopathology observed in the lungs of mice with heterologous viral infections have significant implications for our better understanding of immune-mediated diseases in the human lung. The first important feature was the

dramatic increase in pneumonic consolidation in MCMV-infected influenza A virus-immune mouse lungs. Viral infection-induced lung consolidation has been mainly seen in mice with influenza A virus infection and occasionally parainfluenza virus infection (Yamaguchi et al., 1988) and lethal VV infections (Smee et al., 2001). The consolidation observed in MCMV-infected influenza A virus-immune mouse lung consisted of chronic mononuclear infiltration rather than the acute neutrophilic infiltration observed during acute influenza A virus infection. It is possible that an individual's past history of influenza A virus infections may influence whether they develop more severe chronic consolidation in response to a future infection with a second pathogen. The second feature of interest was the enhanced bronchiolization in MCMV-infected influenza A virus-immune mice. Bronchiolization is a damage-repairing process where the alveolar walls are lined by cells resembling bronchiolar epithelium. This can be observed after a variety of insults such as respiratory infection, exposure to chemical irritants, and even circulatory disturbances involving the lung (Nettesheim and Szakal, 1972). If such modified alveolar tissues are exposed to carcinogens, bronchiolar carcinoma could develop (Nettesheim and Szakal, 1972). The influence of pathogenic viruses such as influenza virus on lung cancers has been indicated (Chang et al., 1984; Kotin, 1966; Nettesheim et al., 1971). Our mouse model suggests that an individual's past history of infections may influence whether they have risk in developing bronchiolar carcinoma in response to an additional antigenic challenge, especially in the presence of environmental carcinogens. Studying this type of model might provide further insights into these conditions.

What is the mechanism of heterologous immunity observed in influenza A virus-immune mice? Since VV and MCMV are large viruses and encode about 200 polypeptides, I hypothesize that VV and MCMV might have epitopes cross-reactive with influenza A virus and those cross-reactive epitopes may stimulate influenza A virus-specific memory T cells upon VV or MCMV infection. VV and MCMV infections also induce potent cytokine responses such as IL-12 and IFN- γ (Biron and Orange, 1995; Cousens et al., 1999; Slezak et al., 2000). In this VV- or MCMV-induced cytokine milieu, it is possible that influenza A virus-specific memory T cells activated by VV or MCMV-encoded cross-reactive epitopes could result in either protective immunity against VV or enhanced MCMV replication. The future studies should be designed to test this hypothesis.

The enhancement of MCMV and LCMV titers in influenza A virus-immune mice is an interesting aspect of heterologous immunity. Antibody-mediated immune enhancement of virus replication, caused by increased uptake of virus through virus-specific antibody complexes by Fc γ R+ cells, has been observed in some viral infections in humans and animal models (Battegay et al., 1993a; Gould et al., 1987; Littaua et al., 1990). The enhanced initiation of viral replication cycles by cytokines such as IL-2 and TNF- α has also been reported in human HIV infection (Kinter and Fauci, 1996; Kitaura et al., 2001a; Kitaura et al., 2001b). Various cytokines such as TNF- α , IL-1, IFN- γ , IL-8 and IL-13 can augment CMV replication in human cells *in vitro*, as well as in rat and mice *in vivo* (Haagmans et al., 1994a; Haagmans et al., 1994b; Hatch et al., 1997; Murayama et al., 1998; Mutimer et al., 1997; Yerkovich et al., 1997). In the present study, the early elevated cytokines such as IFN- γ , IL-1 β , and IL-6 may have contributed

to the enhanced MCMV replication observed in the lungs of MCMV-infected influenza A virus-immune mice. Although the mechanisms involved in cytokine-induced increased MCMV replication are unclear, NF- κ B, which can be activated by pro-inflammatory cytokines such as IFN- γ , IL-1, and TNF- α (Baud and Karin, 2001; Christman et al., 1998), has been shown to activate the CMV enhancer through a viral transactivator (Sambucetti et al., 1989). Furthermore, MCMV replicates in lymphocytes and macrophages (Pottathil et al., 1986; Shanley and Pesanti, 1983). It is conceivable that the massive infiltration with mononuclear lymphocytes and macrophages in MCMV-infected influenza A virus-immune mice lungs might support enhanced replication of MCMV.

Currently, there are few reports available in terms of enhanced LCMV replication: High doses of IL-12 inhibit the development of LCMV-specific CTL; thus they are detrimental to resistance against LCMV (Orange et al., 1994). In future studies examining the cytokine levels in LCMV-infected influenza A virus-immune mice will clarify the role of cytokines in enhancement of LCMV replication by influenza A virus-immunity. In the current study, the observed enhanced mononuclear infiltrates in the lung of influenza A virus-immune mice upon LCMV (clone 13) challenge may result in enhanced LCMV-replication, because clone 13 has an increased ability to replicate in macrophages (Matloubian et al., 1993). In order to study the mechanism involved in this phenomenon, I examined the LCMV-specific CD8 T cell response in LCMV-infected influenza A virus-immune mice. The results from the preliminary experiments showed that the number of LCMV-specific CD8 T cells in LCMV-infected influenza A virus-immune mouse lungs was only half as many as the number of LCMV-specific CD8 T cells in

LCMV-infected control mouse lungs. Future studies should further define whether this altered LCMV-specific CD8 T cell response in LCMV-infected influenza A virus-immune mice contributed to enhancement of LCMV replication and what effector mechanisms were involved in this process.

Heterologous immunity is a new research area and only a few models have been studied. In one study, persistent alveolitis, patchy consolidation and collagen deposition (associated with increased hydroxyproline), upon resolution of virus infection, were reported in Sendai or influenza A virus-infected mouse lungs (Jakab, 1990). Mice that were infected with Sendai virus followed 30 days later by influenza A virus infection had an increase in the persistent alveolitis and hydroxyproline 90 days after the initial infection. Although the author claimed that Sendai virus infection did not alter the replication of influenza virus in the lungs during the acute influenza infection stage, there were around 1 log₁₀ lower influenza virus titers in the lungs of influenza A virus-infected Sendai-immune mice than influenza A virus-infected non-immune mice at each time point they checked. This partial protection is comparable to the heterologous immunity we define. There is one recent paper reporting the successful heterologous protection of C57BL/6 mice from a lethal A/HK/156/97 (H5N1) influenza A virus infection by immunizing first with a serologically distinct H9N2 isolate (O'Neill et al., 2000). This is another example of so-called anti-influenza A virus heterosubtypic immunity (Liang et al., 1994), which is consistent with the concept of heterologous immunity. If a pre-existing pool of memory T cells is activated during infection with a heterologous agent, the T_H1 or T_H2 bias of the memory response might affect the T_H1 or T_H2 bias of the primary response to the heterologous agent. In a murine model of RSV infection, if mice

are infected with influenza A virus before the VV–RSV immunization and RSV challenge, the immune response to RSV is altered and the infection resolves quickly without a strong T_H2 response and serious eosinophilia (Walzl et al., 2000).

The sequence of heterologous virus infections determines the consequences of antiviral immunity and immunopathology. For example, studies with systemic i.p. infections have showed many instances of significant, but not necessarily reciprocal, partial protection. Infection with LCMV, PV or MCMV conferred a considerable level of protection against infection with VV, while VV-immune mice did not have resistance to any of the other viruses (Selin et al., 1998). Similarly, LCMV protected against PV better than PV protected against LCMV (Selin et al., 1998). The lack of reciprocal protection might also relate to whether the potentially cross-reactive T-cell epitope is sufficiently dominant to generate a sizeable pool of memory T cells. In this thesis, I also observed unexpectedly that prior immunity to influenza A virus led to enhanced MCMV and LCMV replication while immunity to influenza A virus still conferred protection against VV. In that murine mouse of RSV infection, prior exposure to influenza A virus reduced lung eosinophilia but did not affect RSV viral load in the lung of rVV-G-primed mice upon RSV challenge (Walzl et al., 2000).

F. New aspects of heterologous immunity in mouse and human diseases

The mice with heterologous virus infections used in this thesis had only been previously exposed to a single virus. The one previous exposure enabled us to relatively easily sort out events occurring during the heterologous viral infection and the possible roles for the factors contributing to heterologous immunity. Our laboratory's ongoing

study has been using the mice that have undergone multiple exposures to several unrelated viruses (with at least 6 week intervals between infections). Those multiple virus-immune mice were then challenged with a virus. Distinct patterns were observed on antiviral immunity against the challenging virus, depending on the history of prior infections with heterologous viruses (Selin et al, unpublished data). For example, As compared to PV challenge of naive mice, immunity to LCMV (single virus-immune) alone protected mice against PV in the spleen. However, immunity to LCMV+**PV** (double-immune) did not protect mice better against PV than did immunity to LCMV alone. Interestingly, prior immunity to LCMV+PV+**VV** (triple-immune) and LCMV+PV+VV+**MCMV** (four-virus-immune) resulted in further reductions of PV titers compared to immunity to LCMV-immune, whereas in the spleen of LCMV+PV+VV+MCMV+**VSV**-immune mice (five-virus-immune), there was an increase in PV titers when compared to the mice immune to three and four viruses. Therefore, the sequence of infections with different viruses determines the consequences of heterologous immunity. Theoretically, these distinct patterns of heterologous immunity in mice immune to multiple viruses may be due to continuous dynamic modulation of the memory T cell pool after each infection.

These observations from experimental models further imply that heterologous immunity in humans might be the determining factor between a clinical and subclinical, or between a lethal and non-lethal, infection. Many viruses in humans—such as Epstein-Barr virus (EBV), and varicella-zoster—cause much more severe infections in teenagers and young adults than they do in younger children (Rickinson and Kieff, 1996; Weinstein and Meade, 1956). Such a difference could be due to immunopathology occurring as a

consequence of the reactivation of memory cells, which may be more diverse and prominent in a more immunologically mature individual. An example of sequential infections in humans inducing severe disease is that of dengue hemorrhagic fever, where different strains of dengue virus express distinct neutralizing antibody epitopes but share highly cross-reactive T cells epitopes (Halstead, 1988). A potent activation of memory T cells by heterologous viruses may result in this enhanced immunopathology (Mathew et al., 1998). Similarly, cross-reactive memory T-cell responses to influenza A virus variants may also become pathogenic to a human population immune to an influenza A virus (Doherty et al., 1997; Jameson et al., 1998). Work in our laboratory has indicated that some T cells that are specific for the HLA-A2-restricted immunodominant BMLF1 peptide of EBV cross-react with the main HLA-A2-restricted immunodominant M1 peptide of influenza A virus even though the peptides have only three amino acids in common. Does such a strong presence of influenza A virus-induced M1-specific T cells in the memory T cell pool predispose the host to severe mononucleosis on EBV infection? Such cross-reactive T cells might also provide enhanced resistance to infection (Welsh and Selin, 2002). A cross-reactive epitope between HCV and influenza A virus was found when individuals that were HCV seronegative were found to generate a putative 'HCV-specific' T-cell response (Wedemeyer et al., 2001), indicating that cross-reactivity may enable a host to generate memory T-cell pools that are specific for a particular pathogen that the host has never experienced. Such a phenomenon could relate to recent findings of HIV-specific T cells in HIV-seronegative individuals who show no signs of harboring HIV (Kaul et al., 2001). It is possible that these cross-reactive HIV-specific T cells may confer

a state of immunity. The field of heterologous immunity is still very new, but the more investigators look, the more examples may be found.

G. Conclusions

Studying heterologous viral infections inoculated via the i.n. mucosal route, I have provided insights into a better understanding of virus-induced activation and modulation of memory CD8 T cells specific for a heterologous virus and their association with heterologous immunity and altered immunopathology in the lung. I have taken advantage of the LCMV and VV, as well as influenza A virus and MCMV systems, and made several novel observations on heterologous antiviral immunity and immunopathology:

1. High frequencies of LCMV-specific memory CD8 T cells, otherwise quite stable, are modulated during a respiratory heterologous VV infection, resulting in (i) an early activation *in vivo* by VV to produce IFN- γ , (ii) a selective expansion and alteration of the original LCMV-specific memory CD8 T cell repertoire, and (iii) distinct organ-dependent redistribution of these LCMV-specific memory T cells.
2. TCR cross-reactivity plays an important role in activation and modulation of LCMV-specific memory CD8 T cells during heterologous VV infections.
3. Virus-induced cytokines play a role in the initial recruitment of LCMV-specific memory CD8 T cells during heterologous VV infections.
4. These modulations of LCMV-specific memory CD8 T cells are associated with protection against a lethal VV challenge and a decreased VV load throughout the course of the acute VV infection. There are altered cytokine profiles and altered lung

immunopathology characterized by prominent lymphocytic and mononuclear infiltrates and the accumulation of LCMV-specific memory CD8⁺ T cells in the expanded BALT.

5. Elevated levels of IFN- γ produced by activated LCMV-specific memory CD8 T cells and enhanced by IL-12 play an important role in mediating the protective heterologous immunity and altered lung immunopathology in VV-infected LCMV-immune mice.
6. The sequence of infections with different viruses determines the outcomes of heterologous viral infections. Upon VV infection, prior exposure to heterologous viruses such as LCMV, MCMV and influenza A virus can be beneficial, resulting in (i) protective immunity against VV, (ii) elevated levels of IFN- γ but suppressed levels of IL-6 or IL-1 β , and (iii) increased lymphocytic and mononuclear responses in the lung.
7. Depending on the infection sequence, prior immunity to influenza A virus can be detrimental, resulting in (i) enhanced MCMV and LCMV replication, (ii) elevated levels of IFN- γ , IL-6, IL-1 β , and IL-12 (upon MCMV infection), and (iii) dramatically enhanced consolidation with prominent lymphocytic and mononuclear infiltrates in the MCMV-infected influenza A virus-immune mouse lungs.
8. Bronchiolitis obliterans in VV-infected LCMV-immune mouse lung and enhanced bronchiolization in MCMV- or LCMV-infected influenza A virus-immune mouse lung may be used as animal models for related human diseases.

The mechanisms that underline some of these important observations remain to be identified. However, this thesis provides a foundation for further examining the role

of memory CD8 T cells in heterologous immunity and immunopathology during respiratory viral infections.

CHAPTER VII

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